Experimental Section

Surface plasmon resonance fluorescence spectroscopy (SPFS): The SPFS binding experiments were carried out with a self-assembled set-up.[16] The CB3(IV) fragment of collagen type IV and the two synthetic heterotrimers were fluorescence-labeled with Cy5 dye (Amersham Pharmacia, Uppsala, Sweden) and the purified human $\alpha1\beta1$ integrin (Chemicon Inc., Temecula, Ca) was embedded into a dimyristoyl phosphatidylethanolamine/phosphatidylcholine bilayer coated onto a gold surface through use of a hydrophilic laminin peptide layer. The binding experiments were performed at 25 °C in tris(hydroxymethyl)aminomethane (Tris)·HCl (50 mM, pH 7.4), NaCl (150 mM), $MgCl_2 \cdot 6H_2O$ (2 mM), and $MnCl_2 \cdot 2H_2O$ (1 mM).

Isothermal titration calorimetry (ITC): ITC measurements were performed at 4 °C on MicroCal VP-ITC equipment by titration of a solution (0.5 μM) of $\alpha1\beta1$ integrin Fos/Jun construct in Tris·HCl (50 mM, pH 7.4), NaCl (150 mM), $MgCl_2 \cdot 6H_2O$ (2 mM), and $MnCl_2 \cdot 2H_2O$ (1 mM) with a solution of each heterotrimer (20 μM) in the same buffer. The data were fitted by using a nonlinear least squares Marquardt algorithm with three independent variables, K_{bind}, ΔH_{bind}, and the stoichiometry N (here fixed as 1);[17, 18] a single set of identical sites was used as the fitting model.

Modeling Experiments: Complex models were created by homology modeling with the program MAIN.[19] Energy minimization was performed starting from the X-ray structure 1QC5[14] of the $\alpha1\beta1$ I domain and a model for the heterotrimers based on the structure of the homotrimer in 1DZI.[12] In addition to the atomic force field, constraints were used to tether homologous Cα positions of the complex model to the 1DZI structure.

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Computational Studies of Subtilisin-Catalyzed Transesterification of Sucrose: Importance of Entropic Effects.

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Introduction

Regioselective acylation of sugars at their hydroxy groups is currently of great importance for the food, chemical, and pharmaceutical industries.[1] Enzymes are very useful for catalysis of these processes owing to their exquisite chemo-, stereo-, and regioselectivity,[2] and subtilisins are among the most researched. One of these specific transformations is the transesterification of sucrose with fatty acid esters with a high selectivity for the 1′-position (Figure 1; experimentally, 80 – 90% of the products are the 1′-product while 2 – 5% are minor species that are largely monoesters whose acylation positions could not be assigned; there is no available thermodynamic data on these reactions).[3]

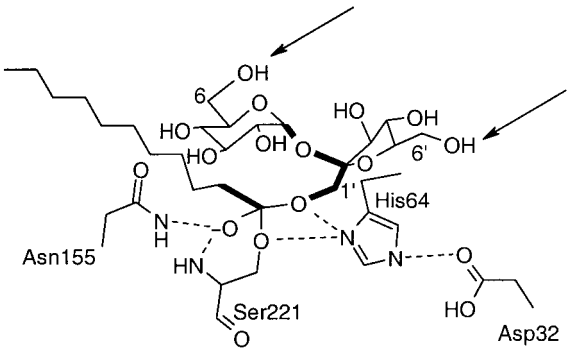

Figure 1. *Schematic representation of the subtilisin active site (catalytic triad and oxyanion hole) and the centres of reactions (the 6OH, 6′OH, and 1′OH sites) in the sucrose moiety. The torsions across which conformational sampling was carried out are indicated by dark lines.*

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Regioselective acylation of carbohydrates leads to a range of starting compounds for the controlled synthesis of a variety of polymers with diverse properties.[4] An understanding of structure – function relationships is therefore desirable. We present here the results of a computational study that examines the origins of this selectivity in the case of sucrose acylation. Previous studies[5] modeled transition-state structures and found that the 1′OH-adduct was preferred to adducts formed at the other OH groups as the 1′OH-adduct was the least sterically constrained. We present the results of a systematic study of this acylation carried out by computation of the free energies of interaction of the possible transition-state adducts and find that in the 1′OH-adduct the sucrose moiety is the most solvent exposed. This exposure confers a higher mobility upon the sucrose, which translates into a larger entropic stabilization. Computation of the enthalpic components of interactions between a protein and its ligands is relatively straightforward and routine. However, computation of entropies has in the past been more difficult, limited by the complexity of the problem and the intensive computations required.[6, 7] We apply a recently developed method that avoids the need for intensive computations and yet seems to capture most of the entropic change in a ligand – protein complex and is thereby attractive for high-throughput computational screenings for lead compounds.[8]

Observations

The conformers with the lowest free energies (as a result of the protein – ligand interactions and the vibrational entropic contribution from the librational modes of the ligand in its "protein cage"[8]) were chosen for analysis. Since we examined the transition states, it is appropriate that the most stable state is one that maximizes the protein – ligand interactions. Our findings are discussed below.

If only the interaction enthalpies are taken into account (Table 1), reaction at the 1′OH-position is marginally favorable, with a stability relative to reaction at 6OH smaller than k*T* (0.7 kcal mol^{-1} at 318 K). In contrast, when we include the entropic components in the free energy, we see that reaction at 1′OH is clearly more favorable than at the other two sites (by about 4 – 5 kcal mol^{-1}).

Van der Waals interactions dominate and account for about 94% of the net interaction enthalpies. Sucrose accounts for 60 – 65% of these interactions while laurate contributes the rest. This domination by van der Waals interactions is not surprising considering that the surface of the ligands is 68% apolar (33% is formed by sucrose and the rest by laurate).

Table 1. *Components of free energy for the lowest-energy conformers.*[a]

Regioisomer	Interaction energy [kcal mol^{-1}]	$T\Delta S$ (T = 318 K) [kcal mol^{-1}]	ΔG [kcal mol^{-1}]
6OH	−62.98 (−6.45, −56.52)	57.41	−120.39
6′OH	−60.64 (−2.90, −57.74)	61.12	−121.76
1′OH	−63.46 (−6.04, −57.41)	61.96	−125.42

[a] Electrostatic and van der Waals components of interaction are given in parentheses.

Table 2. *Contributions from the different ligands to the overall free energy of the protein.*[a]

Regioisomer		Interaction energy [kcal mol^{-1}]	Electrostatics [kcal mol^{-1}]	Van der Waals [kcal mol^{-1}]	Entropy ($T\Delta S$) [kcal mol^{-1}]
	nlau	−24.0	−3.9	−21.1	21.0
6OH	sucr	−39.0	−2.6	−36.4	34.7
	nlau	−20.8	−1.0	−19.8	21.7
6′OH	sucr	−39.9	−1.9	−38.0	34.1
	nlau	−24.2	−3.5	−20.7	21.1
1′OH	sucr	−39.3	−2.5	−36.7	36.7

[a] The entropy values apply at 318 K.

Table 1 reveals that both enthalpy and entropy contribute similar amounts to the net free energy. The discriminating factor between the 1′-adduct and the 6′-adduct is enthalpic, while it is entropic for the 6-adduct. In the 6′-adduct, the electrostatic and van der Waals interactions of the lauroyl moiety are destabilized by approximately 1 – 4k*T*. In contrast, one of the sucrose rings of the 6′-adduct is more buried than in the 1′-adduct and makes electrostatic interactions that are more stable than in the 1′-adduct by about 2k*T* but are offset by van der Waals interactions that are less stable by around 1k*T* (Table 2). In the 6-adduct, the entropic destabilization relative to the 1′-adduct arises largely from the sucrose. The sucrose in the 1′-adduct is slightly more exposed than in the other two adducts and the increase in the relative librational degrees of freedom of the sucrose moiety enhance the stability of the 1′-adduct entropically by about 4k*T*.

In all three adducts, the oxyanion hole caused by the anionic laurate oxygen moiety is stabilized by the side-chain amine group of Asn155 and the backbone amide group of Ser221. In addition, the sucrose makes three hydrogen bonds to the protein backbone atoms and one to the side-chain amine group of Asn155 in the 1′-adduct. Five hydrogen bonds from the sucrose are made to the protein backbone and one to the side chain of Thr220 in the 6′-adduct. Four hydrogen bonds are made to the protein backbone and one to the side chain of Thr220 in the 6-adduct.

The sucrose moiety in both 1′- and 6′-adducts is localized in the S1 pocket of the enzyme, while in the 6-adduct the glucose moiety lies in the S1 pocket and the fructose moiety is in the S3 pocket (Figure 2). The laurate is located near the S2 and S2′ subsites in the 1′-, 6′-, and 6-adducts, respectively.

Graphical inspection of the structures suggests two mutations (possible through site-directed mutagenesis) at sites that are exposed and hence unlikely to cause too much perturbation to the protein structure and stability, and yet may lead to enhanced reactivity at the 1′-adduct: a) Gly127 could be mutated to any neutral residue (Ser/Thr/Asn/Gln) to introduce hydrogen bonds between this amino acid side chain and the hydroxy groups at positions 4 or 6 in the glucose moiety or the 6′-position in the fructose moiety; b) Ser155Asn mutation would increase the length of the side chain and introduce an additional hydrogen bond between this side chain and the hydroxy group at the 6-position of the sucrose. These mutations would, of course, be

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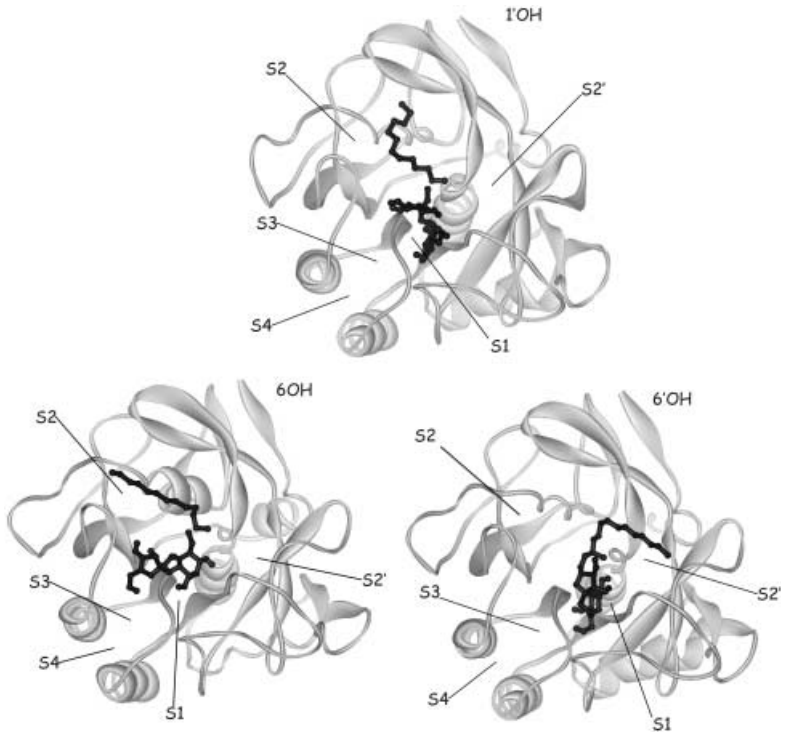

Figure 2. *The minimum vibrational free energy structures for reactions at the three subsites on sucrose. The orientations of the ligands relative to the various subsites of subtilisin are shown.*

subject to some complexity as a result of enthalpy – entropy compensations.[14] One could also mutate destabilizing residues. Val176 is the only amino acid that destabilized the interactions enthalpically (more than 0.1 kcal mol^{-1}). This residue is quite buried in the protein and so any mutation at this site could affect the stability and folding of the protein. We have not come across any reports in the literature that report the effects of mutagenesis on this reaction.

If minimization was discontinued after the gradient of potential was smaller than 10^{-2} kcal mol^{-1} Å^{-1} (as opposed to the 10^{-4} kcal mol^{-1} Å^{-1} cut-off that we actually used for this study), then the computational results indicate that the reaction is most favorable at 6′OH (interaction energies are -61.6, -63.1, and -61.4 kcal mol^{-1} for 6OH, 6′OH, and 1′OH, respectively). This result is contrary to experimental observations and suggests that extensive minimizations are necessary to reach minima that allow for appropriate, more realistic discrimination between the products. However, the discrimination achieved when minimization was discontinued earlier was made on purely enthalpic grounds because in order to compute the entropic contributions minimizations need to be performed until the gradient of potentials is smaller than 0.0001 kcal mol^{-1} Å^{-1}.

In summary, we found that entropic factors are important in determination of the selectivity of the enzyme subtilisin in acylation of sucrose. An appropriate choice of simple and realistic models that take into account the enthalpy of interaction of the ligands and the librational entropy of ligands in their binding pockets can successfully identify the experimentally observed product of the transesterification of sucrose by subtilisin as the lowest energy isomer.

Methods

The enzyme Subtilisin Carlsberg (PDB entry 1cse.pdb, resolved at 1.2 Å)[9] was used as the starting model. Structures that mimic the transition states for reaction at each of the three primary sites on the sucrose (1′OH, 6′OH, and 6OH, see Figure 1) were constructed by using the Quanta software (Accelrys Inc. San Diego). The initial orientations of the ligands were guided by examination of the structures of lipases inhibited with alkylic compounds,[10] while the sucrose was placed such that it was involved in hydrogen bonds with the catalytic triad and with the oxyanion hole (as shown in Figure 1). The Charmm force field[11] was used, with appropriate parameters[12] (electrostatics were modulated through a constant dielectric of 9, which approximately corresponds to the solvent mixture conditions used in such reactions, for example, in the transesterification of disaccharides by using hydrolases[13]), for all calculations. Conformational searches were carried out across 5 torsions (highlighted in Figure 1), by variation of each between the $g-$, $g+$, and t states and minimization of the conformer with decreasing constraints.[12] Minimizations were continued until the gradient of potential energy was less than or equal to 0.0001 kcal mol^{-1} Å^{-1}. We excluded rotations across torsions that would disrupt the oxyanion-hole geometry (the anionic oxygen moiety in Figure 1 is stabilized by the backbone amide group of Ser221 and the side chain amino group of Asn155; the protons on the ring nitrogens of the cationic His64 are hydrogen bonded to the two oxygens atoms that are bonded to the tetrahedral carbon). This process was followed by computation of the vibrational modes of the bound ligand.[8]

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