Analysis of the Interaction of Haloenol Lactone Suicide Substrates with α-Chymotrypsin Using Computer Graphics and Molecular Mechanics

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Abstract: Three-dimensional mapping of the interaction of three haloenol lactone enzyme-activated irreversible inhibitors (suicide substrates) with the active site of α-chymotrypsin has been accomplished by using computer graphics combined with molecular mechanics. According to the proposed inactivation mechanism for these lactones, enzyme inhibition is brought about by a two-step process, i.e., transfer of the lactone acyl group to Ser-195 and subsequent alkylation of His-57 by the resulting halomethyl ketone. In order to study this process, a 115 non-hydrogen atom model of the active site of chymotrypsin was constructed with coordinates taken from an X-ray crystallographic structure, and systematic conformational analysis and energy minimization of the mechanism-based haloenol inhibitors in this model active site were performed at three stages: (1) the non-convalent substrate complex ("Michaelis complex"), (2) the acyl enzyme alkylation complex, and (3) the suicide compound (bis-adduct). The results of these studies support the postulated mechanism, and the calculated energies for the three lactones substrates are in good agreement with the availabile inactivation kinetic data. Estimates of van der Waals interaction energy correlate with the inactivation binding constants (K_i), and estimates of the enthalpy of the alkylation reaction parallel the inactivation rate constants (k_2). In addition, the calculations are also consistent with other known effects of configurational changes and halogen substitution. Such calculations of the interaction between a potential mechanism-based inhibitor and the active site of the target enzyme may be useful in formulating a plausible mechanism of action and in guiding in the synthesis of new, more potent inhibitors.

Recently, we have reported[1] on the synthesis and biological activity of two haloenol lactone systems, 5-halomethylenetetrahydro-2-furanones (I) and 6-halomethylenetetrahydro-2-pyranones (II and III), which act as enzyme-activated irreversible inhibitors of the serine protease α-chymotrypsin. The irreversible inhibition achieved by these suicide substrates or catalytic inhibitors[2] shows excellent potency and efficiency. The general features of the presumed mechanism of action of these lactones are illustrated in Figure 1.

Nucleophilic attack of the serine hydroxyl group at the lactone carbonyl carbon produces an acyl enzyme intermediate; the haloenolate anion, released by the acyl transfer, protonates and ketonizes, forming a halomethyl ketone (A → B). This powerful alkylating agent reacts with suitably positioned nucleophilic residue (Nu in or near the active site (B → C). Alkylation of this residue results in the loss of enzyme activity (C), even if deacylation occurs subsequently (C → D).

We have been interested in assessing the soundness of this presumed mechanism of inactivation and in determining which residue is the site of alkylation. We also hoped to be able to rationalize the rather pronounced differences in the kinetics of chymotrypsin inactivation by these three lactone inhibitor systems and to explore the possibility of increasing the potency and efficiency of their enzyme inhibition.

We have applied computer graphics and molecular mechanics in an attempt to investigate the interactions and the reaction path of three haloenol lactones (I–III in Figure 1) in a model of the active site of chymotrypsin, constructed by using Cartesian coordinates from X-ray crystallographic data and allowing for conformational flexibility of active site residues. The results of these studies support the postulated mechanism, and the calculated energies for the three lactones substrates are in good agreement with the available inactivation kinetic data. Estimates of the van der Waals interaction energy correlate with the inactivation binding constants (K_i), and estimates of the enthalpy of the alkylation reaction parallel the inactivation rate constants (k_2).

Results

Selection of Structures for Study. We have performed conformational analysis and energy minimization calculations on structures that correspond to three stages in the presumed sequence of interactions between the haloenol lactones and chymotrypsin: structure I is the non-covalent substrate complex ("Michaelis complex", leading to the first transition state for acyl enzyme formation); structure II is the acyl enzyme alkylation complex (proceeding to the transition state of His-57 alkylation); and structure III is the suicide compound (the bis-adduct, acylated at Ser-195 and alkylated at His-57). These structures were selected for their significance in the reaction sequence. We have also used them to explore the possibility of obtaining a correlation between the calculated energies and the availabile data on the kinetics of enzyme inactivation. We anticipated that the van der Waals interaction energy (which is estimated from structure I, see below) might correlate with the binding constants for inactivation (K_i) of the different lactones and that from the energies of structures II and III we might be able to obtain an estimate of the enthalpy of the alkylation reaction that might parallel the rate constants for inactivation (k_2).

Construction of the Active Site. The starting geometry of the active site is taken from the X-ray crystallographic structure of toluenesulfonyl α-chymotrypsin.[3] The amino acid residues considered are

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Ala55-Ala56-His57-Cys58-Gly59
S-S-Cys42-Gly43

Asp102

Gly142-Leu143

Ser189-Ser190-Cys191-Met192-Gly193-Asp194-Ser195-Gly196

Val1213-Ser214-Try215-Gly216-Ser217-Ser218-Thr219-Cys220-Ser221

Gly226-Val227-Tyr228

They are the residues that surround catalytic amino acid residues (i.e., Ser-195, Asp-102, and His-57) within a radius of 10 Å. This model of the active site, consisting of 193 non-hydrogen atoms, was minimized by using MAXIMIN,[4] a versatile minimization program, in order to optimize the molecular geometry of the X-ray structure. The root-mean-square differences of the α-carbon of amino acid residue positions between the initial and the minimized structure were 0.10 Å, indicating a modest change in the active site geometry. Due to computational limitations (the program is limited to a maximum of 193 atoms), in the subsequent calculations we decided to use a model of the active site consisting of 115 non-hydrogen atoms, shown in Figure 2. Gly-43, Gly-142, Leu-143, Thr-219, Cys-220, Ser-221, Gly-226, Val-227, Tyr-228, the methylene and carboxyl group of Gly-59, the carboxyl group of Asp-194, and the indole of Trp-215 were removed because they are not facing the three catalytic amino acids and, accordingly, have little chance to interact with the substrate. The amino nitrogens of Ser-218 and α-carbon atoms of Ala-55 and Asp-102 were retained as anchors (i.e., the coordinate of these three atoms were not allowed to change during minimization calculations) to prevent relative movements of the protein backbones. All amino acid residues were treated as conformationally flexible. The receptor site used in the calculations we report in this paper is displayed in Figure 2.

Calculation Methods and Strategy. The calculation of the conformation and energies of structures I–III was done in two stages. First a systematic conformational analysis program (SEARCH)[5] that identifies permissible conformations was used, and then energy minimization was performed on representative permissible conformations. The SEARCH program checks for the van der Waals (VDW) contacts among non-bonded atoms by scanning all possible torsional angles around the rotatable bonds. Generally, one defines several rotatable bonds, the increment of torsional angle Δ, and a VDW radius factor, k. We have used 6 to 10 rotatable bonds, 10° increment of the torsional angle and a k value of 0.90, to allow for thermal vibration of atoms. The collection of discrete sterically allowed conformers constitutes the angle set available to the molecule under study. Obviously, the cardinality of this set depends upon the value assigned to the increment Δ used to explore the conformational space.

Discontinuous resultant angle sets indicate several families of permissible conformations, each continuous angle subset corresponding to one conformational family. Accordingly, a representative conformation from the midpoint of each family of permissible conformations was constructed and then minimized by molecular mechanics calculations. In general, two families were found for each complex: one in which the aromatic ring occupied the hydrophobic pocket, and the other in which the ring pointed away from the active site. Minimization of representative conformers from these two families resulted in an 5–10 kcal/mol differences favoring the aromatic ring within the hydrophobic pocket. This procedure enables one to identify in a systematic way the most stable conformation of the complex molecular system.

Minimization calculations were performed with a versatile minimization program (MAXIMIN).[4] MAXIMIN offers several useful options such as constraint of the geometrical relationship among atoms as an aggregate, constraint of the distance between two specified atoms, or constraint of the absolute coordinates of a set of atoms during the minimization calculation. In this way, we have been able to minimize the docking conformation while maintaining a constant distance between the substrate and the active site consistent with the geometrical constraints imposed by stereoelectronic features of the chemical reaction.

Structure I: Non-Covalent Substrate Complex (Michaelis Complex). The standard bond lengths and angles were used for the (*R*)- and (*S*)-α-phenyl five-membered ring bromoenol lactone I and the (*R*)- and (*S*)-α-phenyl- or naphthyl-substituted six-membered ring bromoenol lactones II and III; the structures were then minimized by the MAXIMIN program. The structure of the complex leading to the transition state for acylation of Ser-195 (structure I) was constructed according to the generally accepted hydrolysis mechanism (see Discussion). Permissible substrate conformations in the enzyme active site (that avoided VDW overlap) were determined with the SEARCH program by systematic rotation around six bonds (Figure 3). We assumed that the carbonyl oxygen of the lactone ring would bind in an analogous manner to other substrates; then within this constraint we sought to determine all plausible binding modes. Therefore, the scheme illustrated in Figure 3 was used for the conformational analysis calculation.

As shown in Figure 3, hydrogen bonding between the lactone carboxyl oxygen and the two amide nitrogens (Gly-193 and Ser-195) was assumed at an optimal distance. The two hydrogen-bonding distance constraints, of 2.76 Å each,[6] compelled the position of the lactone carbonyl oxygen to lie on a circle whose center was equidistant between the two nitrogens (virtual bond *1). Orientation of the substrate relative to the peptide backbone location accounts for the three other angle variables about the virtual bonds *2, *3, and *4. Systematic changes in these four angle variables allowed complete exploration of substrate–enzyme interactional space, while maintaining the geometrical constraints dictated by the hydrogen-bonding assumption.

The virtual four bonds, *1, *2, *3, and *4, connecting the nitrogen of Gly-193 to the carbonyl oxygen of the substrate via three dummy atoms make right angles with one another. The bond length of virtual bond *1 was 2.28 Å, i.e., half-distance between Gly-193 and Ser-195 nitrogens; the other bond lengths, *2, *3, and *4, are 1.56, 0.01, and 0.01 Å, respectively. The dummy atom (Du), which was used instead of the Ser-195 hydroxyl oxygen, prevented the exclusion of possible conformations due to VDW contact between the oxygen and the lactone carbonyl carbon, which were constrained to be close during the SEARCH calculations. In order for this complex to be representative of the molecular species poised for attack of Ser-195 on the lactone carbonyl group (structure I), we assumed a distance of 2.2 Å between the Ser-195 hydroxyl oxygen and the substrate carbonyl carbon, and an angle of 105° between the three atoms Ser---*O*-(H)---*C*=*O*, in accord with the known stereoelectronic constraints in the path of nucleophile approach to a carbonyl group.[7a] This geometry was further supported by force field calculations,[7b] using the methanol–methyl acetate model system for the nucleophilic reaction of Ser-195 with the substrate.

There are 2 176 782 336 conformations theoretically possible for each of the six substrates considered. The SEARCH calculation revealed a number of permissible angle sets for both the *R* and *S* enantiomers of both the five-membered and the six-membered haloenol lactones. The best structure for the conformation of the substrate complex was built up by using the resultant continuous angle sets. The dummy atom was replaced by the real oxygen atom, hydrogens were added to the Ser-195 side chain, and a

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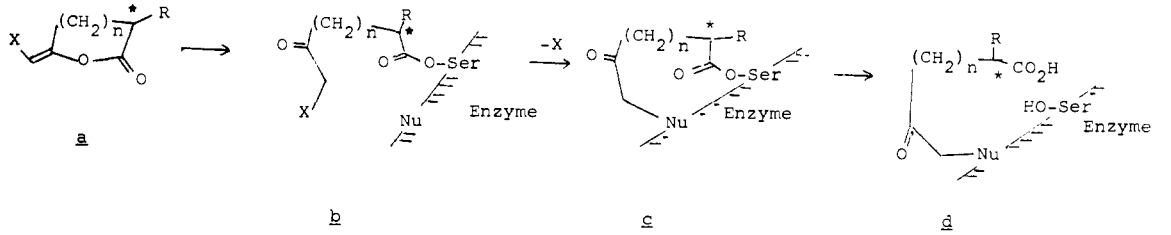

	n	R	X
I	1	Ph	Br, I α-Ph-5
II	2	Ph	Br, I α-Ph-6
III	2	Np	Br, I α-(α-Np)-6

*R and S configurations

Figure 1.

Ala55-Ala56-His57-Cys58-Gly59
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S-S-Cys42

Asp102

Ser189-Ser190-Cys191-Met192-Gly193-Asp194-Ser195-Gly196

Val1213-Ser214-Trp215-Gly216-Ser217-Ser218

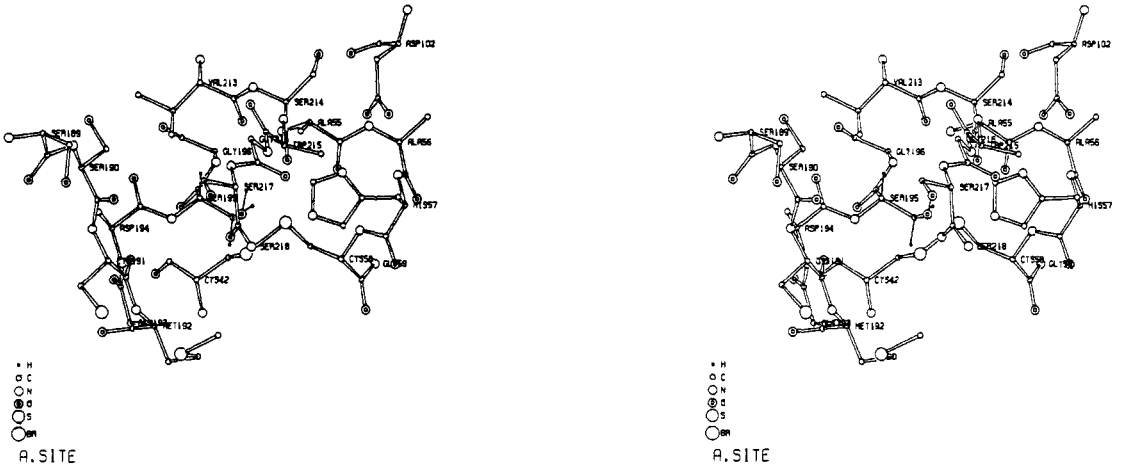

Figure 2. Geometry-minimized model of the α-chymotrypsin active site used in the calculations.

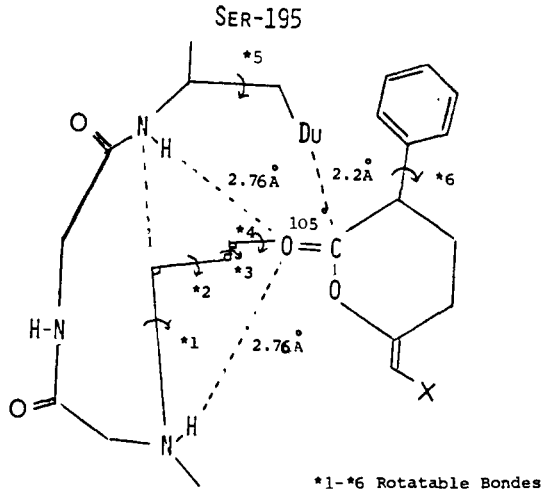

Figure 3. Systematic conformational search.

representative conformation was minimized, preserving the distance between the Ser-195 oxygen and the substrate carbonyl carbon. The number of permissible conformations (i.e., the number of permissible angle sets) and the energies corresponding to the most stable conformer of structure **I** for each compound are shown in Table I.

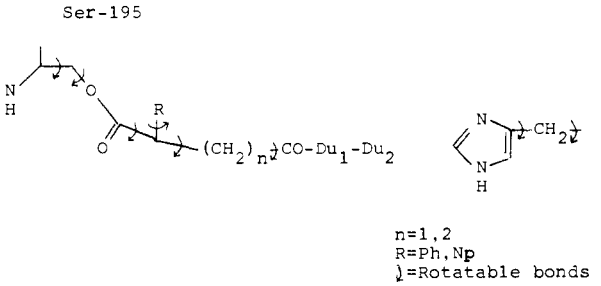

Figure 4.

The information in Table I was used to estimate the van der Waals stabilization energy. This was done by calculating the difference between the energy of structure I and the sum of the energies of the lactone alone (in the same conformation as in structure I, but lacking the surrounding active site) and the active site alone (in the same conformation as in structure I, but lacking the lactone).

Structure II: Acyl Enzyme Alkylation Complex. A structure for the acyl enzyme was constructed, using standard bond lengths and angles (Figure 4), and the halogen atom and methyl group of the halomethyl ketone were replaced by virtual dummy atoms (Du_1, Du_2) in order to prevent exclusion of conformers due to VDW contact between the halomethyl group and the proposed nucleophile. In order for the structure and energy of this complex

Table I. Energy of the Substrate Complex (Structure I) Leading to Ser-195 Acylation by Haloenol Lactones

lactone	energy (kcal/mol) substrate[a] in act. site	act. site	subs.	VDW stabil. energy[b]	permissible conformers found[c]
(*R*)-α-Ph-5 ((*R*)-I)	−22.1	−16.5	3.6	−9.2	224
(*S*)-α-Ph-5 ((*S*)-I)	−22.5	−17.4	4.1	−9.2	816
(*R*)-α-Ph-6 ((*R*)-II)	−26.8	−15.2	−.6	−11.0	8
(*S*)-α-Ph-6 ((*S*)-II)	−29.2	−16.1	−.1	−13.0	320
(*R*)-α-(α-Np)-6 ((*R*)-III)	−27.8	−11.6	1.6	−17.8	4
(*S*)-α-(α-Np)-6 ((*S*)-III)	−35.8	−18.3	.4	−17.9	76

[a] These values include VDW interaction from oxygen of Ser-195 to carbonyl carbon of substrate (+8.5 kcal/mol). [b] van der Waals stabilization energy is given by E(stabilization) = E(substrate in active site)−E(active site)−E(substrate) (see Discussion). [c] Each set of conformers represents two families, i.e., continuous angle space.

Table II. Energy of the Acyl Enzyme Complex for Alkylation of His-57 (Structure II)

lactone precursor	energy[a] (kcal/mol)	conformations found[b]	rotatable bonds
(*R*)-α-Ph-5 ((*R*)-I)	−15.8	134	8
(*S*)-α-Ph-5 ((*S*)-I)	−16.8	928	8
(*R*)-α-Ph-6 ((*R*)-II)	−14.7	1126	9
(*S*)-α-Ph-6 ((*S*)-II)	−12.1	550	9
(*R*)-α-(α-Np)-6 ((*R*)-III)	−19.0	69	9
(*S*)-α-(α-Np)-6 ((*S*)-III)	−14.7	23	9

These values include VDW interaction energy from nitrogen to carbon of halomethyl group (5.5 kcal/mol). [b] Each set of conformer represents two families, i.e., continuous angle space.

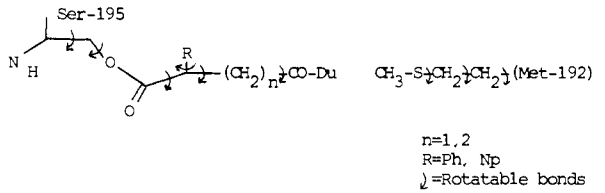

Figure 5.

to represent that of an acyl enzyme poised to approach the transition state for alkylation of His-57, we performed the search for permissible conformations with a distance constraint of 2.28 Å from His-57 nitrogen (the first candidate nucleophile) to the Du_1 atom. This represents the shortest distance which maintains the repulsive VDW interactions between the imidazole and the halomethyl hydrogens below 1 kcal/mol. The carbonyl group was maintained perpendicular to the imidazole ring, as is required by stereoelectronic considerations in this displacement reaction (see Discussion). As with the acylation reaction, the final conformations for structure II were built up by using representative permissible conformations from the SEARCH angle sets and replacing the dummy atoms by the corresponding real atoms. These structures were then minimized, preserving the distance between the histidine nitrogen and the carbon of halomethyl group. The number of conformations found and the refined conformation energies for structure II are summarized in Table II.

We also calculated the energy for the acyl enzyme complex leading to the reaction of the halomethyl group with the second candidate nucleophile, Met-192 sulfur (Figure 5). Hydrogens were added to the side chain of Met-192, and the SEARCH calculations were carried out under the distance constraint of 2.2 Å from sulfur to the Du atom. The final structures were built up with representative permissible conformations from the resultant angle subsets; the dummy atom was replaced by the halomethyl group, and the completed structure was minimized, preserving the distance of 2.2 Å between sulfur and carbon atom of halomethyl group. For the structures that represent the reaction of the acyl enzymes derived from (*S*)-α-Ph-5 ((*S*)-I) and (*R*)-α-Ph-6 ((*R*)-II) with Met-192, we obtained 10 kcal/mol and 15 kcal/mol, respectively. (The values include a VDW interaction between the sulfur and halomethyl carbon, i.e., 21 kcal/mol.)

Structure III: Suicide Compound (Bis-Adduct: Ser-195 Acylated and His-57 Alkylated). We assumed standard geometry for the suicide compound. Permissible conformations were identified by using the same model as for the alkylation reaction (Figure 4). The SEARCH calculation was carried out with the distance constraint of 1.45 Å from the nitrogen of imidazole to Du_1 atom, and the resultant number of permissible conformers and conformational families found are given in Table III. The dummy atom was then replaced by the real carbon atom, and the additional bond connecting the nitrogen to carbon atom was considered in order to simulate the alkylation. Finally, the hydrogens were added to Ser-195, His-57, and substrate, and the conformations were minimized to give structure III. The results are summarized in Table III.

Table III. Energies of the Suicide Compound (Bis-Adduct; Structure III)[a]

lactone precursor	energy (kcal/mol)	no. of conformers found	no. of conformational families
(*R*)-α-Ph-5 ((*R*)-I)	−22.0	37752	5
(*S*)-α-Ph-5 ((*S*)-I)	−24.0	56	2
(*R*)-α-Ph-6 ((*R*)-II)	−26.4	11868	4
(*S*)-α-Ph-6 ((*S*)-II)	−23.0	4	2
(*R*)-α-(α-Np)-6 ((*S*)-III)	−33.3	8	2
(*S*)-α-(α-Np)-6 ((*S*)-III)	−28.1	2	2

[a] We used 8 and 9 rotatable bonds for the five- and six-membered ring compounds, respectively.

Discussion

The chymotrypsin suicide substrates we have studied were considered to follow the generally accepted hydrolysis mechanism[8] depicted in Figure 6, although the hydrogen bonding of the carbonyl oxygen shown in Figure 6, structure **1**, may not occur in peptide hydrolysis as a crucial ingredient of catalysis until the transition state **2**. The acyl enzyme intermediate **3** results from the reaction between the Ser-195 hydroxyl group and the carbonyl group of the substrate; in the case of the haloenol lactones, this intermediate possesses the very reactive halomethyl ketone alkylating moiety. The suicide complex is produced by the reaction of this moiety with a suitably positioned nucleophilic residue in the active site of chymotrypsin.

Using molecular mechanics calculations and computer graphics, we have attempted to evaluate the energies of three structures: structure I, the noncovalent substrate complex (Michaelis complex, leading to the first transition state for acyl enzyme formation); structure II, the acyl enzyme alkylation complex (proceeding to the transition state of the His-57 alkylation reaction); and structure III, the suicide compound (the bis-adduct, resulting from acylation at Ser-195 and alkylation at His-57).

Structure I: Non-Covalent Substrate Complex (Michaelis Complex). The catalytic amino acid residues of α-chymotrypsin, His-57, Asp-102, and Ser-195, are involved in two hydrogen bonds, namely, between the hydroxyl group of serine and the nitrogen of imidazole and between the amino hydrogen of imidazole and

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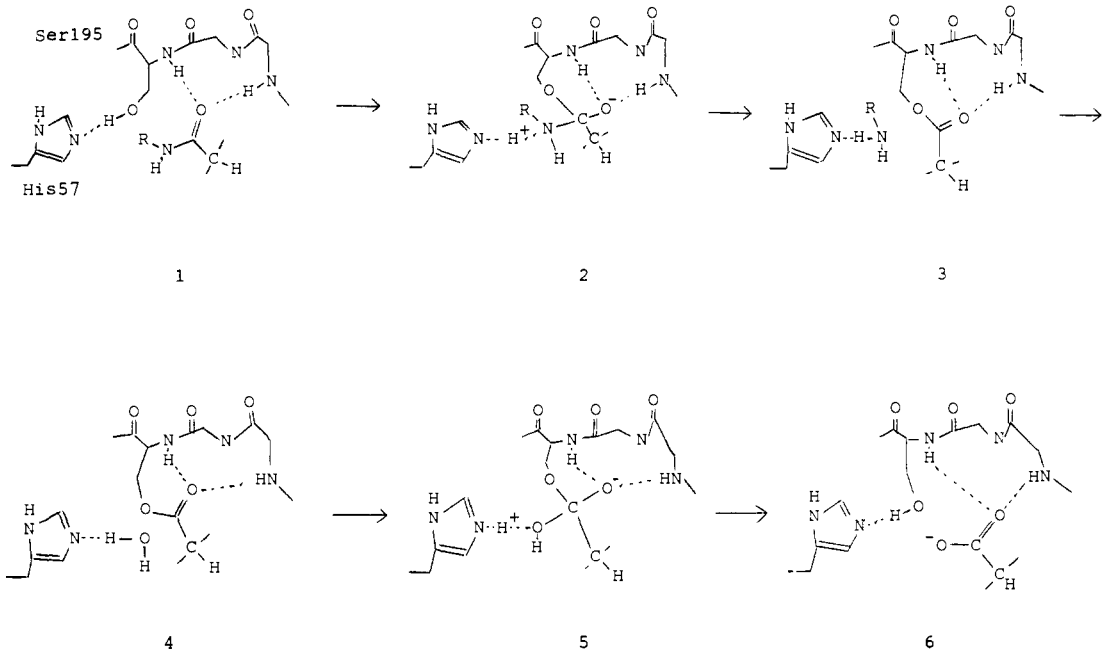

Figure 6. Hydrolysis mechanisms.

the carboxylic acid oxygen of Asp-102, respectively. When the suicide substrate approaches the active site, it is anchored by a hydrogen bond between its carbonyl oxygen and nitrogen atoms of Ser-195 and Gly-193 (Figure 6, structure **1**). Subsequently, nucleophilic attack by the oxygen of Ser-195 produces the tetrahedral intermediate shown in Figure 6, structure **2**, which is converted immediately to the acyl enzyme (Figure 6, structure **3**).

Recently, Scheraga,[10] Detar,[11] Durant,[12] and Kollman[13] have reported the conformational analysis of several peptide substrates in the α-chymotrypsin active site. These peptide substrates are involved in *three* hydrogen bonds with the peptide backbone: the Gly-193 and Ser-195 nitrogens with the substrate carbonyl oxygen as well as the Ser-214 carbonyl oxygen with the substrate amino nitrogen. These authors have analyzed by molecular mechanics calculations the conformation of the acylation transition state, the tetrahedral intermediate proceeding to the acyl enzyme, and the resulting acyl enzyme. They also discussed the activity differences between *d*- and *l*-optical isomers. The conformational analysis of suicide substrates reported in this paper is more complex and more difficult than that of the peptide substrates: our lactone substrates have much greater conformational flexibility because they lack the third hydrogen bond (Ser-214–substrate amino group), which in the case of peptides suggests a specific docking conformation to be minimized. Thus, as a necessity we have had to make systematic analyses of all docking conformations in order to identify the most stable one.

In calculating the energy of the noncovalent substrate complex (Michaelis complex, structure I), we have retained the two hydrogen bonds between the amino groups of Ser-195 and Gly-193 and the carbonyl oxygen of substrate, and we have specified a slight interaction between the serine oxygen and the carbonyl carbon of substrate, as these centers are poised for undergoing the acylation reaction (Figure 6, structures **1** and **2**). To do this, we have used the following geometrical parameters: 2.76 Å for the length of hydrogen bond between amino group of Ser-195 and Gly-193 and carbonyl oxygen of substrate[6], a distance of 2.2 Å between the nucleophilic oxygen and carbonyl carbon, and an angle of 105° between nucleophilic oxygen and carbonyl group. The latter parameter values were based on our force field calculations[7b] of the interaction of methanol with methyl acetate, which we have considered as a simple model system for this reaction, and they are consistent with the results of Dunitz and Bürgi,[7a] who studied the geometry of nucleophile approach to carbonyl systems. Corresponding to this geometry, the repulsive VDW interactions between the nucleophilic Ser-195 hydroxyl group and the three adjacent atoms (i.e., the α-carbon and the lactone ring carbonyl oxygens) amounts of less than 1 kcal/mol. (Shorter distances bring about strong repulsion, e.g., 37 kcal/mol for a distance of 2.0 Å.) This conformation resembles closely the conformation reported by Durant for a similar system.[12]

The results of computations on the Michaelis complex (structure I) are displayed in Table I. The refined stable conformations of the (*S*)-α-Ph-six-membered ring and the (*R*)- and (*S*)-α-(α-Np)-six-membered ring lactones shown in Figure 7, parts a–c, respectively, clearly reveal a beneficial interaction between the substrate phenyl or naphthyl group and the hydrophilic cleft of the active site. The other substrates also exhibit this same hydrophobic interaction between the phenyl or naphthyl group and the active site.

Energy calculations performed on the substrate without the surrounding active site and on the active site without substrate included were used to provide an estimate of the VDW energy difference before and after docking the substrate in the active site (van der Waals stabilization energy). This energy gives an *approximate* measure of the enthalpy of substrate binding (and of reaction free energy, if one assumes that entropic differences are small because all of these substrates access the active site in a very similar manner, cf. Figure 7). As shown in Table IV, the calculated van der Waals stabilization energies for the Michaelis complexes (structure I) are in good agreement with the inactivation dissociation complex K_i, especially if one considers that the solvent and entropy were assumed not to have a major influence.

The substrate–receptor complex is stabilized by about 9 kcal/mol in the case of five-membered lactones, and in the case of six-membered lactones by about 12 kcal/mol (phenyl) and 18 kcal/mol (naphthyl). Independent force field calculations show that naphthalene is stabilized by about 5 kcal/mol more than benzene, due to increased VDW interactions with the active site. Accordingly, this accounts for all but ca. 1 kcal/mol of the energy difference between the phenyl- and naphthyl-substituted six-membered ring compounds.

The calculations indicate very little difference in the interaction energies of (*R*)- and (*S*)-enantiomers, a fact that is confirmed

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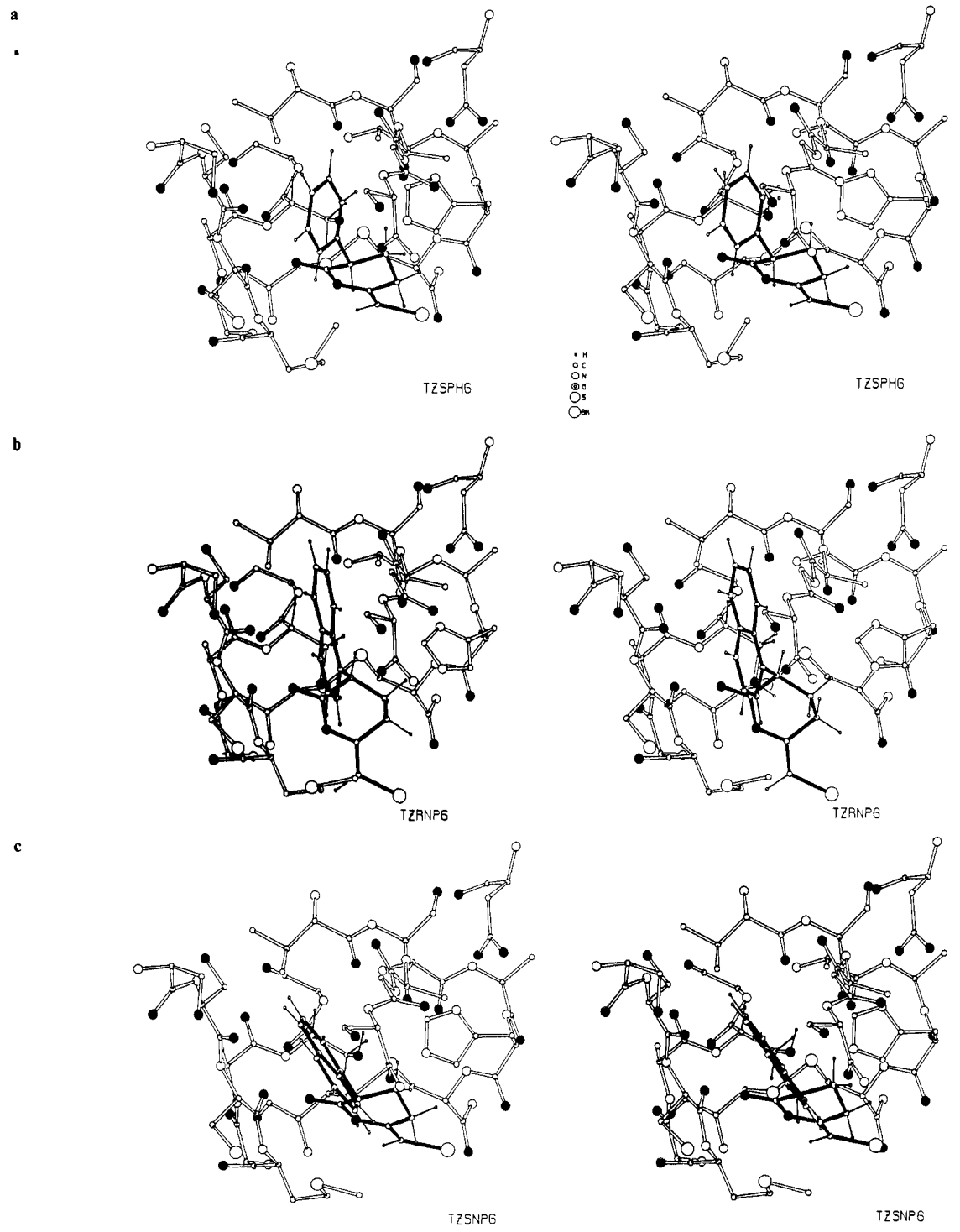

Figure 7. Structure I: substrate complexes of S-α-Ph-6 (a; S-II), R-α-(α-Np)-6 (b; R-III), and S-α-(α-Np)-6 (c; S-III).

in the two cases studied (cf. Table IV). Further, in agreement with data collected in Table IV, Figure 7 shows that the bromine is located outisde the active site cavity, and thus, the substituent located at this position makes only a minor contribution to the enzyme–substrate interaction.

We have explored the topography of the enzyme active site further by calculating the VDW energy difference before and after docking the (*S*)-α-(β-Np)-six-membered lactone. The calculated interaction of −17.6 kcal/mol compares well with the measured K_i value for (*R*,*S*)-α-(β-Np)-six membered lactones of 0.0734,[9] thus, within experimental and computational error, the α- and β-naphthyl-substituted compounds show the same activity. The calculations also clearly indicate that there is a large space available in the hydrophobic pocket that could accommodate even bulkier hydrophobic substituents, which suggest that lactones with lower K_i values (i.e., increased affinity) could be made.

It is worth mentioning that we have applied our computational procedure to the peptide substrate AcNH–Trp–OCH_3, including the third hydrogen bond between the amino group of tryptophan and the Ser-214 carbonyl oxygen; the calculations lead to almost the same conformation as the one reported by Wipff et al.,[13] with the indole group of tryptophan located in the hydrophobic area of the active site.

Structure II: Acyl Enzyme Alkylation Complex. The non-covalent substrate complex (Michaelis complex) discussed above proceeds by Ser-195 hydroxyl addition to give a tetrahedral intermediate which is subsequently converted by elimination, protonation, and ketonization of the haloenolate ion to the acyl enzyme. This species, which contains the halomethyl ketone moiety, can partition between two reaction paths. If a water molecule

Table IV. Comparison of Calculated and Measured Interaction Energies of Haloenol Lactones with Chymotrypsin

lactone	substrate interaction energy (kcal/mol)[a]	K_i (μM) obsd[b] X = Br	X = I	
(*R*)-α-Ph-5 ((*R*)-I)	−9.2			
(*S*)-α-Ph-5 ((*S*)-I)	−9.2	(50.0)	(42.2)	
(*R*)-α-Ph-6 ((*R*)-II)	−11.0			0.218
(*S*)-α-Ph-6 ((*S*)-II)	−13.0	(0.298)	(0.172)	0.131
(*R*)-α-(α-Np)-6 ((*R*)-III)	−17.8			0.0388
(*S*)-α-(α-Np)-6 ((*S*)-III)	−17.9	(0.0978)	(0.0566)	0.0783

[a] Interaction energies are taken from Table I (see Discussion). [b] For the inactivation binding constant (K_i), values in parentheses are for racemic mixtures and have been taken from ref 1; those for the six-membered ring lactones (II and III) have been corrected by a factor of 1/6.5. This correction is needed because an erroneous value for the K_m of the competing substrate Ac–Trp–*p*–NO_2–phenyl ester (2 μM instead of 0.31 μM) was originally used. Values for individual enantiomers are from ref 9.

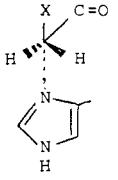

Figure 8.

hydrogen bonded to the imidazole (His-57) nitrogen attacks the acyl carbonyl, this will produce a tetrahedral intermediate (Figure 6, structure **5**) that can eliminate the Ser-195 hydroxyl group, releasing the hydrolized lactone and regenerating the free enzyme (catalytic turnover). Alternatively, if the halomethyl ketone reacts with a nucleophilic residue located in or near the active site before catalytic turnover, then the enzyme is permanently inactivated. This partitioning between turnover and inactivation determines the inactivation efficiency of a haloenol lactone (i.e., its turnover per inactivation number).

Among the candidate nucleophiles, Asp-102 is unlikely because of steric hindrance, but the imidazole nitrogens of His-57 or the sulfur atom of Met-192 represent good candidates. We have simulated the approach to the transition-state conformation for an S_N2 displacement on the halomethyl group[14] by these two nucleophiles (acyl enzyme alkylation complex, structure II) as follows.

For the attack on His-57 (Figure 8), conformational analysis calculations were carried out by using the SEARCH program with a distance constraint of 2.28 Å between the imidazole nitrogen and the carbon atom of the halomethyl group (α-carbon). In addition, the imidazole nitrogen was constrained to approach the α-carbon atom from a direction opposite of that of the leaving group (backside attack), and the imidazole ring of the histidine was constrained to be in a plane perpendicular to that of the carbonyl group (orbital overlap).[14] There is a stereoelectronic reason for this latter constraint. α-Halo ketones are especially good alkylating agents because the trigonal bipyramidal structure of the S_N2 transition state can be stabilized by a π-type interaction with the p-orbitals of the carbonyl group, a situation that can operate only when the carbonyl group is maintained in a plane perpendicular to the axis defined by the halogen, the α-carbon, and the imidazole nitrogen.[14] Energy-refined calculation results are summarized in Table II. Parts a and b of Figure 9 display the conformations thought to approximate the complexes leading to the transition states for this reaction with the lactones (*S*)-II and (*S*)-III.

Table V. Energy Difference between the Acyl Enzyme Alkylation Complex and the Suicide Compound

lactone	energy diff (kcal/mol)	k_2 (min^{-1})[a] X = Br	X = I	
(*R*)-α-Ph-5 ((*R*)-I)	−6.2	(0.0355)		
(*S*)-α-Ph-5 ((*S*)-I)	−7.2			
(*R*)-α-Ph-6 ((*R*)-II)	−11.7	(8.59)	(7.22)	7.94
(*S*)-α-Ph-6 ((*S*)-II)	−10.9			6.83
(*R*)-α-(α-Np)-6 ((*R*)-III)	−14.3	(7.69)	(6.99)	6.37
(*S*)-α-(α-Np)-6 ((*S*)-III)	−14.9			7.29

[a] Inactivation rate constant (k_2). Numbers in parentheses are for racemic mixtures (from ref 1), other values are from ref 9.

The approach to the transition state involving the halomethyl group and Met-192 was calculated by using the SEARCH program and the distance constraint of 2.2 Å between the sulfur of Met-192 and the carbon on the halomethyl group. The energies of these alkylation complexes were nearly 10 kcal/mol higher than those for the complexes leading to the reaction with His-57, making Met-192 unlikely as the site of alkylation. In agreement with this inference is the fact that these lactone substrates will still inactivate modified chymotrypsins in which Met-192 is converted to the (*S*)-methylsulfonium salt[15] or the sulfoxide.[16] Therefore, we have examined only the complex leading to the alkylation reaction of His-57 by the halomethyl ketone group (Table III). Figure 10 illustrates the conformation of the bis-adduct with lactone (*S*)-II having the phenyl group located in the hydrophobic area of the active site.

Structure III: Suicide Compound (Bis-Adduct). The energy differences between the conformation of the acyl enzyme alkylation complex (structue II) and the conformation of the final suicide compounds (bis-adduct (structure III)) may provide an approximate measure of the reaction enthalpy for the alkylation reaction, if the entropic contribution to the free energy can be neglected. The acyl enzyme alkylation complex corresponds to the beginning of the reaction (approach to the transition state) and the suicide compound corresponds to the product. Inspection of Table V shows that the reactivity of the phenyl-substituted five- and six-membered ring lactones (I and II) is correctly reproduced by using this simplistic energy difference approach, and the predicted small energy differences between enantiomers are consistent with the VDW stabilization energy calculations (Table I) and the views of the substrate complex (Figure 7), which pictured the active site as a spacious hydrophobic pocket. The reactivity of naphthyl-substituted six-membered ring lactones (III), however, is greatly overestimated.

We attempted to identify the factors which may have caused this discrepancy by using computer graphics in connection with the molecular mechanics calculations reported in Table V. Careful examination of the spatial location of the phenyl group during the reaction showed the same small movement (less than 2 Å) for the four lactones ((*R*)- and (*S*)-I and (*R*)- and (*S*-)-II); by contrast, the naphthyl group in (*R*)- and (*S*)-III shows a larger movement (about 4 Å) during reaction. Thus, it seems plausible to conclude that entropy makes a different contribution to the reaction free energy in the case of the phenyl- vs. the naphthyl-substituted molecules. Accordingly, it seems appropriate to neglect the entropy contribution *within each* series of molecules (i.e., (*R*)- and (*S*)-α-phenyl-substituted five- and six-membered lactones or (*R*)- and (*S*)hα-(α-naphthyl)-substituted six-membered ring lactones, respectively), but *not within the whole* series.

Conclusion

Molecular mechanics calculations and computer graphics analysis of the interaction of three haloenol lactone suicide inhibitors with a model of the active site of α-chymotrypsin have

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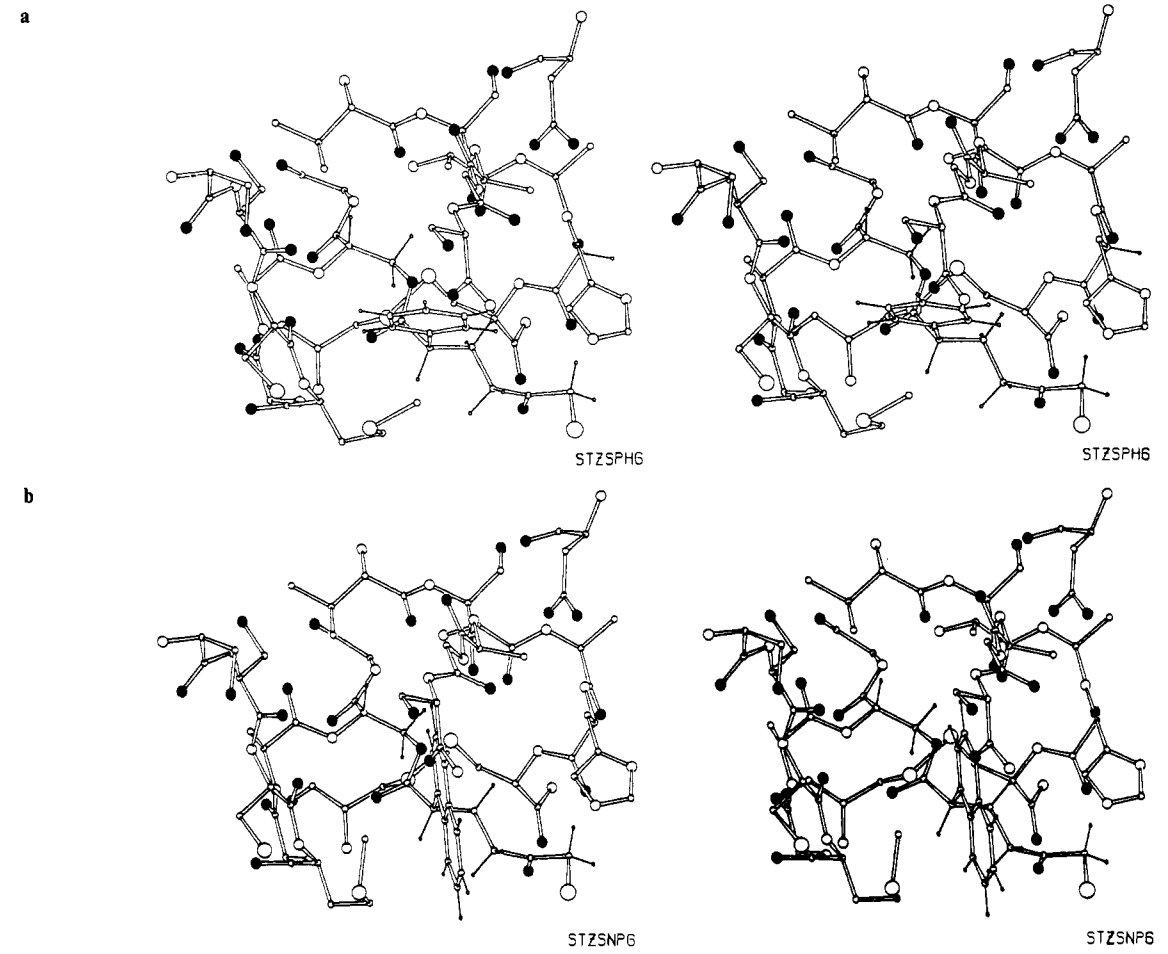

Figure 9. Structure II: acyl enzyme alkylation complexes of S-α-Ph-6 (a; S-II) and S-α-(α-Np)-6 (b; S-III).

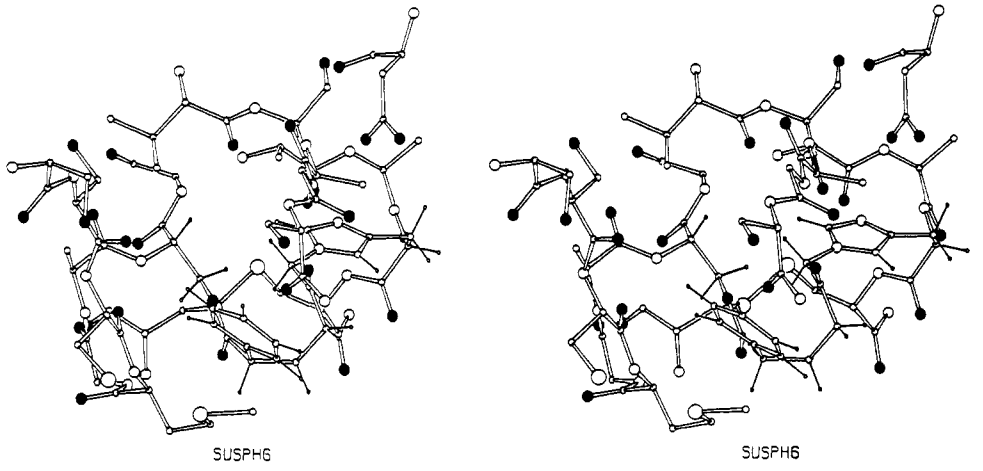

Figure 10. Structure III: suicide compound of S-α-Ph-6 (S-II).

demonstrated that such an approach is useful in understanding substrate–active site interactions. These suicide substrates approach the active site and produce an acyl enzyme which alkylates the His-57 residue, inactivating the enzyme.

Three key stages of this interaction have been modeled: structure I, the non-covalent substrate complex (Michaelis complex) leading to the first transition state for acyl enzyme formation; structure II, the acyl enzyme alkylation complex (proceeding to the transition state of His-57 alkylation); and structure III, the suicide compound (the bis-adduct, acylated at Ser-195 and alkylated at His-57). The acylation process with these substrates has proven to be similar to the acylation with peptide substrate. As an alkylation site, we considered both the His-57 nitrogen atom and, alternatively, the Met-192 sulfur atom; the calculations have clearly shown that His-57 is the most probable candidate, since the complex leading to the alkylation of His-57 is about 10 kcal/mol more stable than that leading to the alkylation of Met-192.

We have investigated the non-covalent substrate complex (structure I) in a systematic manner, and in subsequent minimization calculations we have shown that the phenyl and naphthyl groups occupy the hydrophobic pocket of the enzyme site. The van der Waals stabilization energy (taken as the energy difference before and after docking the substrate in the active site) exhibits good parallelism to the value of the inactivation binding constant (K_i). Furthermore, the energy differences between the alkylation complex (structure II) and the suicide compound (structure III) did correlate with the rate of enzyme inactivation (k_2). The

calculations also confirm the relatively small differences in bioactivity of optical isomers.

Thus, our computational procedure offers an approach to evaluate alternative mechanisms and to rationalize observed bioactivities; it, as well, offers guidance for the design of new potent suicide inhibitors. A noteworthy finding in regard to the last point is that there is a large space available in the hydrophobic pocket that could accommodate yet bulkier and more hydrophobic substituents. While such molecular alterations might be beneficial to the binding characteristics of these lactones (K_i), it might be detrimental to their rate of inactivation (k_2). The conflicting effect brought about by such changes in substrate size and shape should be properly considered in designing new catalytic inhibitors.

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Supplementary Material Available: Figures of three non-covalent substrate complexes (structure I), four acyl enzyme alkylation complexes (structure II), and six suicide compounds (structure III), as well as the coordinates for all figures (50 pages). Ordering information is given on any current masthead page.

Communications to the Editor

Supermonomolecular Structure in the Langmuir-Blodgett Films of a Surface-Active Dye-Fatty Acid Mixed System

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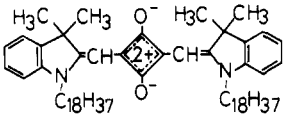
A few studies have appeared on the construction of a supermonomolecular layer, defined as a complex layer upon the surface of water that involves more than one monolayer to form a supermonomolecular structure.[1-3] It will be very useful in expanding the technique of Langmuir-Blodgett (LB) films to construct such a supermonomolecular layer that is capable of being deposited on a substrate to form a unique structure.

We have found that a layer obtained under higher pressure, so far considered as a "collapsed" film, retains an ordered structure and that the deposition of this film results in an LB film with a unique bilayer unit structure. We also found that it is now possible to prepare and transfer either one monolayer or a bilayer of a surface-active dye-fatty acid mixed system merely by the control of the surface pressure (Π). Preliminary results are given here.

The synthesis of a squarylium dye, 2,4-bis[(3,3-dimethyl-1-octadecyl-2(3*H*)-indolylidene)methyl]-1,3-cyclobutadienediylium-1,3-diolate (**1**) was accomplished by a modified



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procedure in the literature.[4] Eicosanoic acid (**2**) was purchased from Eastman Kodak Co. and used without further purification. Π-*A* isotherms were measured by using a Lauda Filmwaage on an aqueous solution of 4.0×10^{-4} M $CdCl_2$ and 5.0×10^{-5} M $KHCO_3$ at 17 °C and pH 6.0. Chloroform was used as a spreading solvent. The thickness of LB films was determined by the stylus method. The instrument used was a Taylor-Hobson Talystep with a tip radius of 12.5 μm and the stylus force was 2 mg.

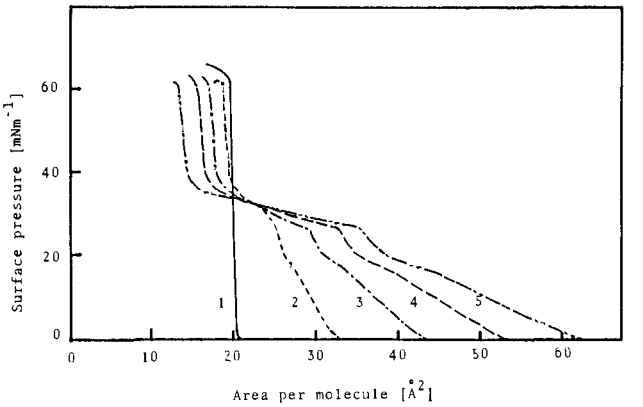

Figure 1. Surface-pressure isotherms of monolayers curves 1-5; surface pressure vs. area per **2** molecule (curve 1) and mixed films of **1** and **2** of molar mixing ratio *r* (*r* = 1:10, curve 2; *r* = 1:5, curve 3; *r* = 1:3, curve 4; *r* = 1:2, curve 5).

Π-*A* isotherms of mixed monolayer of **1** and **2** with molar ratios ranging from 1:10 to 1:2 showed that two "collapse" points (by the conventional explanation[5]) are seen at 30 and up to 60 mN m^{-1} (Figure 1). We assumed that these points represent the occurrence of two condensed states and calculated the area of **1** at 25 and 40 mN m^{-1} on the assumption that the area of **2** per molecule of mixed monolayer is the same as in pure monolayer. The calculated area per molecule of **1** is 70 $Å^2$ and nearly 0 $Å^2$ at 25 and 40 mN m^{-1}, respectively. The latter value is unusual in view of the fact that **1** has two long alkyl chains. However, we can rule out the possibility of **1** being dissolved into the subphase. The addition of NaCl ranging from 1×10^{-3} to 5×10^{-3} M also afforded the same values for the areas per molecule of **1** at both 25 and 40 mN m^{-1}. In addition, for the case of *r* = 1:5, the optical density of **1** at 640 nm (λ_{max} for the main peak) of the deposited films at 40 mN m^{-1} (HPF) was about twice those of the films at 25 mN m^{-1} (LPF). These results show that a mixed monolayer is formed at 25 mN m^{-1} and that **1** is squeezed out from the monolayer of **2** at around 40 mN m^{-1} to form a complex layer, whose Π-*A* behavior is governed by **2** alone.

Mixed films of **1** and **2** (*r* = 1:5) were deposited at 25 and 40 mN m^{-1} on a slide glass as typical Y films, and in each case the apparent transfer ratios for both the downward and the upward strokes were between 1.0 and 1.1. The values of electric capa-

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