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The Oxyanion Hole in Serine β-Lactamase Catalysis: Interactions of Thiono Substrates with the Active Site[1]

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Both functional and structural studies of serine β-lactamases indicate the existence of an oxyanion hole at the active site with an important role in catalysis. The functional presence of the oxyanion hole is demonstrated by the previous observation that thiono-β-lactams are very poor substrates of β-lactamases (B. P. Murphy, and R. F. Pratt, 1988, *Biochem. J.* **256,** 669–672) and in the present paper by the inability of these enzymes to catalyze hydrolysis of a thiono analog of a depsipeptide substrate. This thiono effect was first noted and interpreted in regard to classical serine hydrolases although the chemical basis for it has not been firmly established either in those enzymes or in β-lactamases. In this paper a computational approach to a further understanding of the effect has been taken. The results for a class C β-lactamase show that the deacylation tetrahedral intermediate interacted more strongly with the enzyme with an O^- placed in the oxyanion hole than an S^-. On the other hand, the converse was true for acylation tetrahedral intermediate species, a result distinctly not in accord with experiment. These results indicate that the thiono effect does not arise from unfavorable interactions between enzyme and thiono substrate at the tetrahedral intermediate stage but must be purely kinetic in nature, i.e., arise in a transitional species at an early stage of the acylation reaction. The same conclusion as to the origin of the thiono effect was also indicated by a less extensive series of calculations on a class A β-lactamase and on chymotrypsin.

Key Words: oxyanion hole; thiono effect; serine hydrolase; β-lactamase; molecular mechanics.

INTRODUCTION

The concept of an "oxyanion hole" has by now achieved quite a long history in discussions of the catalytic mechanisms of serine and cysteine hydrolases. The term seems to have been introduced by Henderson who observed in his crystal structure of a stable acyl-chymotrypsin a pair of backbone NH hydrogen-bond donors closely adjacent to the carbonyl oxygen of the acyl group. He proposed that such hydrogen bonds to the substrate might be stronger in the transition state than in the ground state and thereby an important factor in catalysis (*1*). Subsequently a similar arrangement of hydrogen bond donors or, equivalently, dipoles, has been observed in the crystal

[1] This paper is dedicated to Professor Thomas C. Bruice on the occasion of his 75th birthday.
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structures of all serine and cysteine hydrolases and, termed the "oxyanion hole," is now a central feature of descriptions of the catalytic mechanisms of these enzymes (*2–4*). In strong support of this general idea, the crystal structures of complexes between these enzymes and transition state analog inhibitors that generate tetrahedral oxyanions on interaction with the active site serine or cysteine nucleophile usually exhibit anionic occupation of the oxyanion hole (*2–3*).

A structural effect, believed to derive from the interaction of the oxyanion hole with bound substrates, has been directly observed in favorable cases, and attempts have been made to measure the concomitant functional effects. For example, resonance Raman and infrared absorption spectra have provided evidence that the carbonyl bonds of acyl-serine proteinases are lengthened, as would be expected if the carbonyl oxygen were a hydrogen-bond acceptor, and to an extent correlated with hydrolytic reactivity (*5–8*). The expression of the phenomenon in normal catalysis has been detected and quantitatively evaluated by measurement of the effects of perturbations on the structure of substrates (*9–11*) and the enzyme (*12–16*). Model hydrogen-bonded systems have been constructed to estimate the catalytic effect of the oxyanion hole (*17*) and theoretical methods have also been employed (*18–20*).

Another now well-known functional manifestation of the oxyanion hole in the serine and cysteine hydrolases is the effect of replacing oxygen with sulfur in the carbonyl group of the scissile bond. Campbell *et al.* (*21*) demonstrated that this transformation led to a large decrease, some 2000-fold, in k_{cat}/K_m (bimolecular acylation) with specific ester substrates of chymotrypsin and subtilisin. Smaller decreases were measured with less specific substrates. Similar effects on deacylation (k_{cat}) were observed. Polgár and co-workers (*22,23*) reported comparable results for k_{cat}/K_m values of these enzymes. The latter authors also noted that the effect was not present in ester hydrolyses catalyzed by a variety of cysteine proteinases, and in particular papain, although a crystal structure of the latter had suggested the presence of an oxyanion hole (*24*). The papain result was extended to a greater variety of ester substrates by Storer and co-workers (*25,26*) who also observed that a significant decrease due to thiono substitution *did* occur in k_{cat} values for papain.

These observations have been interpreted to mean that the incorporation of sulfur rather than oxygen into an oxyanion hole is less favorable and leads to diminished catalysis. The effect is greatest in the most specific substrates where the complete catalytic machinery is mobilized. The importance of the latter point with respect to papain has been emphasized by Foje and Hanzlik (*27*) who showed that specific thiono *amides* were not detectably hydrolyzed by papain.

Two distinctly different explanations for the thiono-substitution effect have been offered in the above-mentioned literature sources:

(a) The larger sulfur alters the position of the substrate with respect to the catalytic apparatus (the oxyanion hole *and* other catalytic functional groups) to one less optimal for catalysis.

(b) Hydrogen bonds to sulfur are intrinsically weaker than to oxygen and thus the oxyanion hole is a less effective catalyst. A related idea is that S^- (being less basic than the analogous O^-) requires less stabilization by hydrogen bonding and thus an oxyanion hole has less effect.

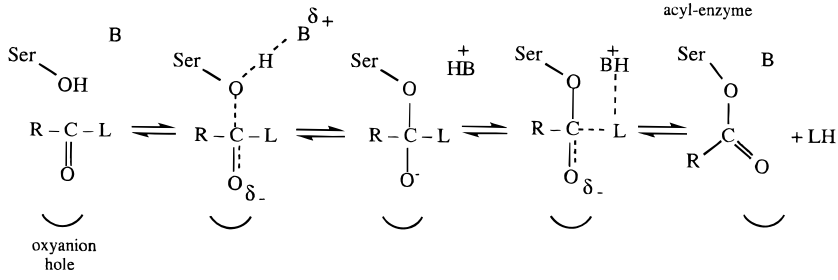
These alternatives differ in that, by comparison with the oxygen analog, (a) involves

a nonoptimal interaction with the active site whereas (b) suggests an optimal but less effective interaction. If the relevant transition states could be examined, one would expect to see misalignment of the active site and substrate (again, in comparison with the oxygen analog) in the case of (a) but not in (b). In both cases, however, interaction energies between active site and transition state might be expected to be smaller in the thiono analog.

A largely unanswered question is that of at what stage along the reaction coordinate is the effect of the oxyanion hole maximally expressed (*21*,*28*,*29*). We assume that the turnover of a substrate by a serine hydrolase involves first the acylation sequence shown in Scheme 1. This includes an anionic tetrahedral intermediate (shallow energy minimum), two flanking transition states (energy maxima) each with considerable tetrahedral oxyanion character, and the acyl enzyme (energy minimum). Deacylation is generally represented as the reverse of this sequence with water replacing LH. In Scheme 1, B represents the general base catalyst of the active site. One might imagine, for specific substrates at least, that the maximal effect of the oxyanion hole would be exerted at the transition state (or states in the case of a double-displacement mechanism) and less at the Michaelis complex and acyl enzyme stages, but to what extent would it be found in the tetrahedral intermediate(s)? This would presumably depend on the degree of similarity between the transition state and the tetrahedral intermediate.

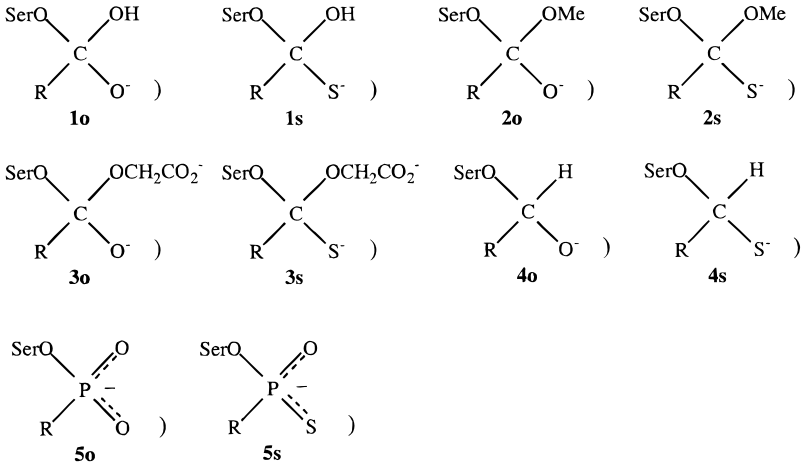
Some years ago, the thiono-substitution test ("S test") was employed in this laboratory to demonstrate the presence of a catalytically important oxyanion hole in serine β-lactamases and a closely related DD-peptidase (*30*). These enzymes also catalyze an acyl-transfer reaction by a double-displacement mechanism involving an acyl-enzyme intermediate (*31*,*32*). Crystal structures reveal active sites both similar and different to those of serine proteinases but an oxyanion hole is clearly indicated (*33*–*35*).

Recently we have used computational methods to judge the ability of tetrahedral anionic adducts to interact with the active site of the class C β-lactamase of *Enterobacter cloacae* P99 (*36*). As a logical extension of that work we present here a comparison between the series of oxo and thiono adducts **1–5** in order to obtain insight into any differences that there might be between the interactions of oxo and thiono species with this active site at the tetrahedral intermediate stage and thus further address the



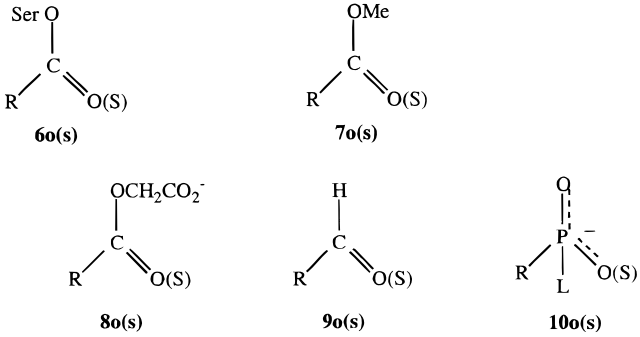
SCHEME 1

question of the molecular basis of the S test. In the structures **1–5,** SerO represents the active site serine residue that is covalently bound to the ligand, R represents the β-lactamase-specific side chain $PhCH_2CONHCH_2$, and) represents the oxyanion hole; the remainder of the active site, the enzyme, and the surrounding solvent are not represented. Of the species **1–5,**



STRUCTURE 1

1 represents the tetrahedral intermediate formed on hydrolytic deacylation of the acyl enzyme **6, 2** represents the tetrahedral intermediate formed on acylation of the enzyme by the nonspecific substrate **7, 3** that on acylation by the more specific (bearing a carboxylate-containing leaving group (*37*) substrate **8, 4** the tetrahedral adduct between the enzyme and the aldehyde **9,** and **5** the transition state or tetrahedral intermediate analog adduct formed on phosphorylation of the enzyme by the inhibitor **10,**



STRUCTURE 2

where L is a good leaving group. In each instance, energy-minimized structures were obtained and are discussed here in terms of active site structure and interaction

energies between enzyme and tetrahedral ligand. Correlation with experiment was achieved by means of the substrates **8o** and **8s.** The method was also extended in less detail to the class A β-lactamase of *Staphylococcus aureus* PC1 and to chymotrypsin. The overall conclusion is that there appears to be no evidence of a general mechanism of destabilization of tetrahedral thiolate anions with respect to oxyanions in the oxyanion hole and therefore the thiono effect must have more subtle kinetic origins.

MATERIALS AND METHODS

N-(Phenylacetyl)thionoglycylglycolic acid **8o** was prepared from reaction of H_2S with an appropriate imidate ester (*40*). *N*-(Phenylacetyl)glycylglycolate was available from previous studies in this laboratory (*41*). The β-lactamase of *Enterobacter cloacae* P99 was obtained from the Centre for Applied Microbiology & Research (Porton Down, Wiltshire, UK). The chromophoric substrate PADAC {7-(thienyl-2-acetamido)-3-[2-(4-*N*,*N*-dimethylaminophenylazo)pyridinium-methyl]-3-cephem-4-carboxylic acid} was purchased from Calbiochem. The inhibition kinetics were performed at 25°C in 20 mM Mops buffer at pH 7.5. Absorption spectra and spectrophotometric rates were obtained from either a Perkin-Elmer Lambda 4D or a Hewlett-Packard HP8452 spectrophotometer. Inhibition constants were obtained from measurements of the effect of the inhibitor on the rate of enzyme-catalyzed PADAC hydrolysis under $S_0 << K_m$ conditions (enzyme and PADAC concentrations were 16 nM and 16 μM, respectively).

The hydrolysis of **8o** and **8s** in 50 mM sodium bicarbonate in ^2H_2O was monitored by ^1H NMR by means of a Varian Gemini 300 spectrometer. Concentrations of depsipeptides and enzyme were 4 mM and 25 μM, respectively.

Computational Methods

The computations were set up essentially as described previously *(37).* The starting point was the crystal structure of the P99 β-lactamase with a phosphonate inhibitor covalently bound to the active site serine residue **(5o)** and the computations were performed by means of an IBM 530H computer with INSIGHT II, version 2.20 (Biosym Technologies Inc., San Diego, CA). Other tetrahedral ligand structures **(1–5)** were obtained by direct replacement by means of the Builder module of INSIGHT II. It was assumed that standard force field parameters could be applied to each of the tetrahedral species constructed. The initial conformations of the ester alkyl groups of **2** and **3** were extended away from Ser 64 O_γ—initial dihedral angles O_γCOC and COCC were $-170°$ in each case. Atomic charges on the protein were as assigned by INSIGHT II. The phenolic oxygen of Tyr 150, apparently hydrogen-bonded to Ser 64 O_γ in the crystal structure *(39),* was protonated while Lys 67 and Lys 315 were cationic, as required by the generally accepted mechanism of action of this enzyme *(34,39).* The charges on the inhibitor were generated by MNDO calculations (MOPAC 6.0) on a *N*-acetylglycylserylisoleucinamide tripeptide with the ligand bound to the serine O_γ. A list of the important charges and bond lengths employed is given in Table 1. The enzyme complex was then dynamically equilibrated with a 15 Å sphere of water centered at the active site serine O_γ and the energy of the system minimized, without constraint, within a CV force field by means of 1000 steps by the method of steepest descents and 2000 steps of conjugate gradients. The final derivative of

TABLE 1

Atomic Charges and Bond Lengths Assigned to the MXY^- Moiety in the Tetrahedral Complexes **(14)** of Ligands with the P99 β-Lactamase

	Atomic charge			Bond length (Å)	
Complex	M	X	Y	MX	MY
1o	0.453	−0.427	−0.740	1.41	1.43
1s	0.248	−0.355	−0.754	1.40	1.71
2o	0.448	−0.439	−0.690	1.44	1.44
2s	0.276	−0.350	−0.714	1.44	1.71
3o	0.488	−0.382	−0.733	1.45	1.45
3s	0.288	−0.337	−0.768	1.44	1.71
4o	0.402	−0.089	−0.732	1.09	1.43
4s	0.153	−0.046	−0.771	1.00	1.77
5o	1.168	−0.728	−0.754	1.61	1.61
5s	0.828	−0.713	−0.613	1.60	2.00

energy with respect to structural perturbation was then in the range of 0.05–0.08 kcal/mol-Å. Interaction energies, including all nonbonded interactions, could be obtained directly from the minimized structure by means of the DISCOVER program within INSIGHT II. Residues included in E_{int} calculations for the P99 β-lactamase were Ser 64, Lys 67, Tyr 150, Asn 152, Lys 315, Thr 316, Gly 317, and Ser 318, for the PC1 β-lactamase, Ser 70, Lys 73, Ser 130, Asn 132, Glu 166, Asn 170, Lys 234, Ser 235, Gly 236, Gln 237, and Ala 238, and for chymotrypsin, His 57, Asp 102, Gly 193, and Ser 195. Although it is probable that longer range interactions might influence the absolute value of calculated interaction energies and conceivably even their relative values, the major aim of the present work was to examine the effects of the local environment and in particular the residues involved in catalysis. Previous results with the P99 β-lactamase indicated that long-range interactions did not discriminate between tetrahedral adducts (*36*).

The Builder module of INSIGHT II was also used to construct substrate complexes **15** and **16** along a Bürgi-Dunitz trajectory (*42*,*43*). Initially, prior to energy minimization, the (thiono)carbonyl group was directed into the oxyanion hole. The Ser 64 O_γ was protonated while the Tyr 150 hydroxyl group was deprotonated. The latter would be expected for the active form of the enzyme if this residue did perform the role of a general base in the first step of catalysis (*34*,*39*). A fixed distance of 2.5 Å between the Ser 64 O_γ and the substrate (thio)carbonyl carbon atom was employed and fixed angles of either 126° **(15)** or 135° **(16)** between a line between the above-mentioned atoms and one in the plane of the substrate carbonyl group bisecting the C−C(=X)−O angle. The O_γ and C=X atoms were also fixed in space in order to hold the trajectory position throughout the minimization procedure.

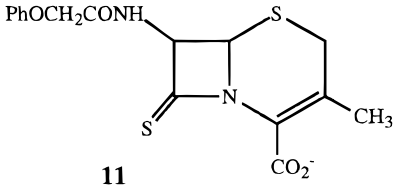
The same procedures were employed for the PC1 β-lactamase and chymotrypsin. Structural coordinates for the PC1 phosphonate complex were obtained from Dr. Osnat Herzberg (*44*) and those for monoisopropylphosphoryl chymotrypsin were obtained from the Brookhaven Data Bank (Filename 1gmh). Both Glu 166 and Lys 73 were in their protonated (acidic) form for the calculation, as they would be

irrespective of which was the general base facilitating formation of the tetrahedral intermediate. For the chymotrypsin calculation, His 57 was protonated (cationic) and Asp 102 anionic.

RESULTS AND DISCUSSION

The ^{1}H NMR spectra and spectrophotometric assays showed that although **8o** was a substrate of the P99 β-lactamase, yielding the expected phenylacetylglycine and glycolate as products, its thiono analog **8s** was not, to any detectable degree. The depsipeptides **8o** and **8s** are both weak reversible inhibitors of the P99 β-lactamase with K_I values of 11 and 5.5 mM, respectively. Since **8s** is not a substrate of the enzyme, this K_I presumably reflects noncovalent binding. The latter is likely to be true also for **8o** since, for alkyl esters, enzyme acylation is probably rate determining at substrate saturation (*45*) and thus $K_I = K_m = K_s$.

The results described above suggest that the response of serine β-lactamases to a thiono substrate closely resembles that of other typical serine hydrolases, viz. the thiono analogs of specific oxo substrates are very poor or nonsubstrates. They extend the earlier finding that the cephalosporin **11** is, relative to its oxo analog, a very poor substrate of class A and C β-lactamases (*30*). Despite the presumably greater potential flexibility of the acyclic depsipeptide at the active site, the thiono analog still does not react. These results, by analogy with the classical serine proteinase situation, can be interpreted in terms of a functioning oxyanion hole in serine β-lactamases.



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STRUCTURE 3

Enzyme–Ligand Interactions—Structure

In an attempt to learn more of the structural basis for this thiono effect, computer models of the tetrahedral ligands **1–5** at the active site of the class C β-lactamase of *E. cloacae* P99 were constructed as described under Materials and Methods. In this approach, the various different chemical species were prepared by suitable element substitution in a computer-model derived from the crystal structure of the phosphonate derivative **5o** *(39)*; the new species was hydrated by a molecular dynamics method and then allowed to proceed toward an energy minimum by a molecular mechanics procedure. Active site features of the final structures achieved are given in Table 2, where the distances listed are shown in Fig. 1. In the original crystal structure of **5o,** the distances B, C_1, E, J, L_1, and L_2 seemed short enough to represent hydrogen-bonding situations. In particular, it seemed that a neutral Tyr 150 hydroxyl group was hydrogen-bonded to the Ser 64 O_γ oxygen (distance C_1) and to the Lys 67 ammonium ion (distance B). Energy minimization in the CV force field led, as described previously *(36)*, to the movement of Tyr 150 to a position closer to a phosphonyl oxygen than

TABLE 2

Active Site Distances (Å) in the Ligand Complexes with the P99 β-Lactamase after Energy Minimization

	Distance[a]												
Complex	A_1	A_2	B	C_1	C_2	D	E	G_1	G_2	J	K	L_1	L_2
5o[b]	3.32	5.32	2.77	3.01	3.40	3.46	2.57	4.70	4.31	2.71	3.24	3.05	2.29
1o	2.78	4.33	3.79	3.08	2.56	2.88	2.75	4.75	4.30	3.08	3.15	3.19	2.77
1s	2.82	4.42	3.98	3.23	2.63	3.01	2.79	4.82	4.09	2.94	3.22	3.39	2.90
2o	3.52	5.49	3.94	2.70	3.78	3.55	2.90	4.80	4.91	3.08	3.92	3.60	2.86
2s	2.92	4.63	4.07	3.09	2.64	3.27	2.93	4.84	4.03	2.94	2.93	3.58	2.91
3o	3.06	4.94	4.23	2.85	3.68	3.32	2.90	4.83	4.87	3.18	3.59	3.26	2.86
3s	2.79	4.29	3.65	3.16	2.62	3.62	2.79	4.99	3.98	2.96	3.19	3.40	2.74
4o	2.78	—	4.67	3.40	—	3.24	2.82	5.37	—	3.25	3.12	3.28	2.79
4s	2.87	—	5.04	3.23	—	2.89	2.89	5.26	—	3.08	2.92	3.47	2.90
5o	2.89	4.64	3.74	3.25	2.45	3.31	2.85	4.49	3.29	3.15	2.92	3.84	2.86
5s	2.78	4.54	4.07	3.26	2.46	2.91	2.71	4.90	4.48	3.12	3.15	3.58	2.74

[a] Distances refer to Fig. 1.
[b] Crystal structure distances.

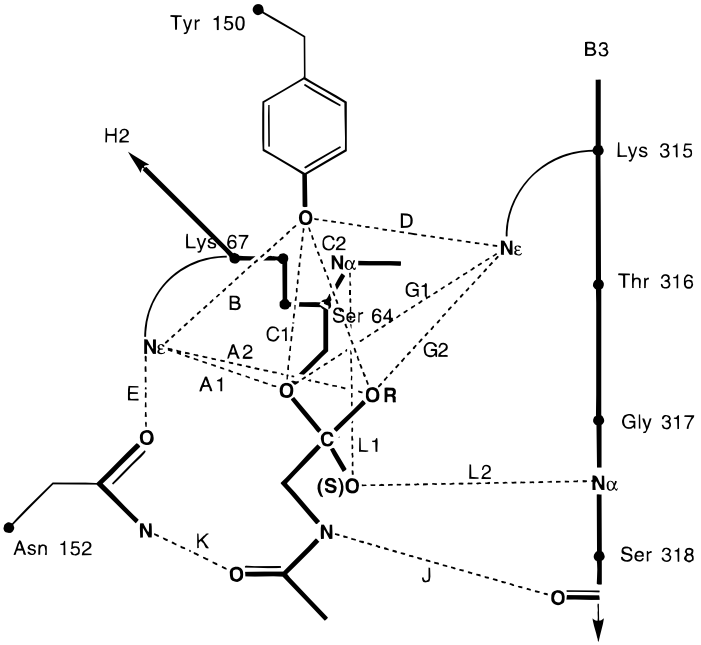
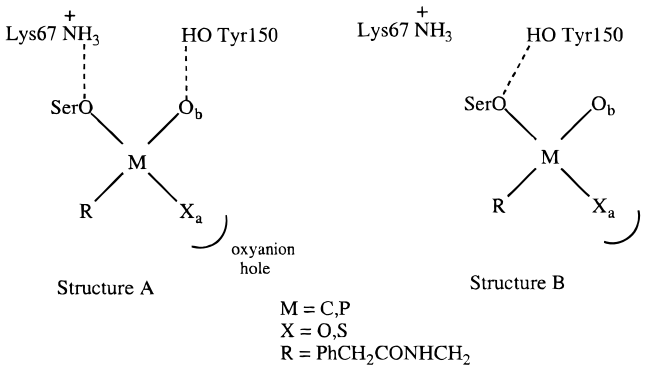


FIG. 1. Schematic diagram of a tetrahedral intermediate at the active site of the P99 β-lactamase. The substrate (bold lines) is attached to Ser 64 at the C-terminus of helix 2 (bold line, H2), adjacent to β-strand 3 (bold line, B3). Side chains of Lys 67, Asn 152, Tyr 150, and Lys 315, which interact with the substrate, are also depicted. Bold points represent the α-carbons of amino acid residues and Nα the backbone nitrogen atoms comprising the oxyanion hole. The distances listed in Table 2 are indicated by letter-labeled dashed lines.



SCHEME 2

to Ser O_γ and of the ammonium ion of the Lys 67 into a hydrogen-bonding situation with Ser O_γ. This conformation will be henceforward referred to as Structure A (Scheme 2) and is represented below and shown for **5o** in detail in Fig. 2 of the previous paper (*36*). A structure apparently very similar to A was also attained by **5s,** the thiono analog of **5o** (Table 2). In particular, the positioning of the S^- in the oxyanion hole seems very similar to that of O_a^- in **5o.** In all of the energy-minimized structures, both in the present and in the former paper, and indeed in the original crystal structure, the interaction of the oxyanion with the backbone NH of Ser 318 was always stronger than with that of Ser 64 ($L_1 > L_2$). In all cases the oxyanion also held an attendant water molecule at hydrogen-bonding distance.

Structures similar to A, as defined particularly by the positions of Tyr 150 and Lys 67 with respect to Ser O_γ and O_b, were attained by **1o** and **1s,** the carbon-centered pair most closely resembling the phosphonyl derivatives. As noted in the introduction, these represent tetrahedral intermediates in the deacylation of an acyl enzyme and thiono-acyl enzyme, respectively. Occupancy of the oxyanion hole is again very similar. The Tyr 150 hydroxyl appears hydrogen-bonded to the ammonium ion of Lys 315 in both cases, which is also a feature of **5s.** In the aldehyde adducts, **4o** and **4s,** which lack O_b, the Lys 67 ammonium ion has also moved into hydrogen-bonding distance of Ser O_γ.

In the structures described until this point, there seemed very little that was significantly different between the oxo and the thiono analogs. A rather striking difference between these variants appears, however, when the acylation tetrahedral intermediates **2** and **3** were considered. As is clear from Table 2, the oxo and thiono compounds, despite identical initial conformations, lead to different energy-minimized structures, and consistently for **2** and **3.** In both **2** and **3,** the thiono derivative after energy minimization has achieved Structure A. In contrast, the oxo analogs took up a structure (denoted Structure B, Scheme 2) where the Tyr 150 hydroxyl group is hydrogen-bonded to Ser O_γ and Lys 67 remains further away. The two structures, A and B, corresponding to **2s** and **2o,** respectively, are shown in greater detail in Fig. 2.

The position of the leaving group carboxylates in **3o** and **3s** is of interest. Both

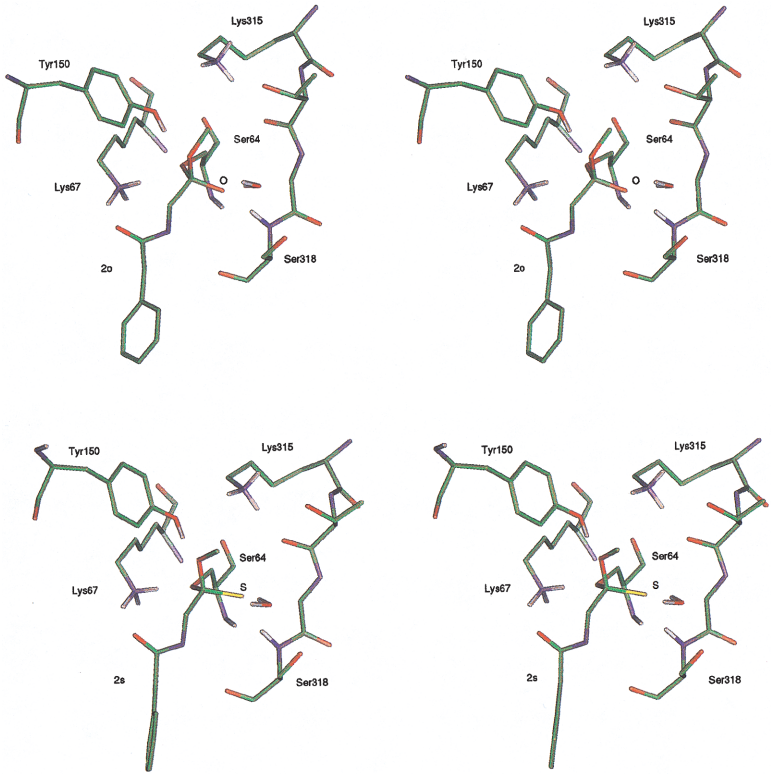


FIG. 2. Energy minimized structures corresponding to P99 β-lactamase acylation tetrahedral intermediates **2o** (upper) and **2s** (lower), derived from the substrates **7o** and **7s,** respectively. The substrate moiety is shown with the termini of the side chains of the important amino acid residues Lys 67, Tyr 150, and Lys 315, the NH components of the oxyanion hole, and the water molecule generally found close to $O(S)^-$.

are projecting into solvent and appear hydrogen-bonded to a number of water molecules. The somewhat different orientation of the inhibitor in the two cases, which correlates with the differences denoted above by structures A and B, brings the carboxylate of **2o** closer to the side chain oxygen of Thr 316 than to the side chain nitrogen of Lys 315 while vice versa is true of **2s.** In the latter case, the Lys 315 ammonium ion appears to be hydrogen-bonded to one of the carboxylate oxygen atoms.

Enzyme–Ligand Interactions—Energy

Previous experience (*36*) has suggested that a good measure of the relative binding effectiveness of a series of tetrahedral anionic ligands with the P99 β-lactamase can be derived from consideration of the noncovalent energies of interaction, E_{int}, given by equation 1,

$$E_{\text{int}} = E_{\text{E}} + E_{\text{I}} + E_{\text{EOI(noncov)}} \qquad [1]$$

between the ligands and eight important residues of the active site. In Eq. [1], E_{E}

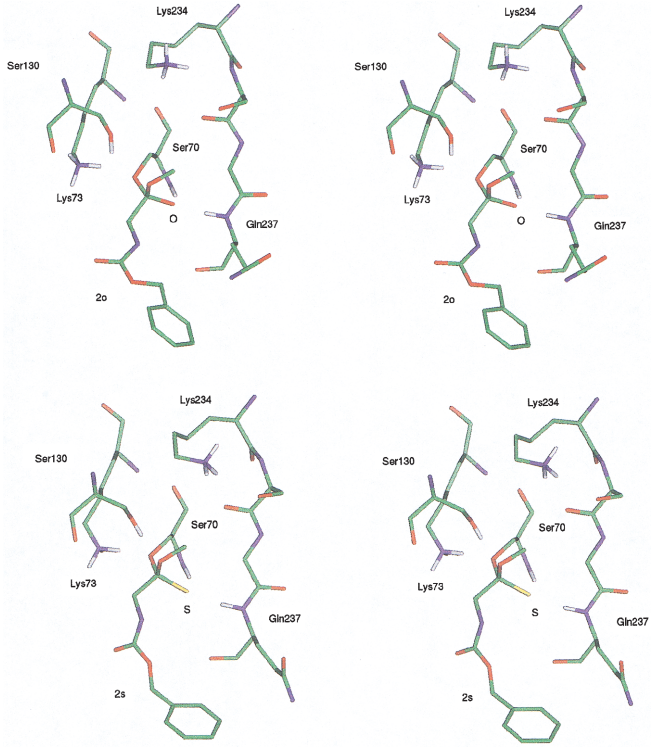


FIG. 3. Energy-minimized structures corresponding to PC1 β-lactamase acylation tetrahedral intermediates **2o** (upper) and **2s** (lower), derived from the substrates **7o** and **7s,** respectively. The substrate moiety is shown with the termini of the important amino acid residues Lys 73, Ser 130, and Lys 234, and the NH components of the oxyanion hole.

and E_I represent the internal energies of the enzyme and bound ligand, respectively, these terms including contributions from deviations from ideal geometry and from internal nonbonded interactions, and $E_{EOI(noncov)}$ represents the noncovalent interaction energies between enzyme and ligand. These values for **1–5** are given in Table 3.

The first noticeable feature of the E_{int} values, for structure **1,** is that the oxo analog **1o** interacts with the enzyme more strongly than the sulfur **1s.** This would suggest that the enzyme would stabilize **1o** more strongly than **1s** which might be thought to correlate with experiment. It should be noted, however, that the tetrahedral species **1** represent tetrahedral intermediates in the *deacylation* step in turnover of oxo and thiono substrates. Since no turnover of thiono esters such as **8s** is observed, nor any significant enzyme inhibition by **8s,** there is no evidence that acyl enzymes such as **6s** are actually formed from thionoester substrates; i.e., it is likely that the experimentally observed problem with thiono substrates arises in the *acylation* step and thus the behavior of the acyl enzyme is experimentally inaccessible. In the case of thiono-β-lactams such as **11,** slow turnover does occur, but this may well reflect slow, rate-determining enzyme acylation. Certainly the R61 DD-peptidase is only very slowly, if at all, acylated by **11** (*30*).

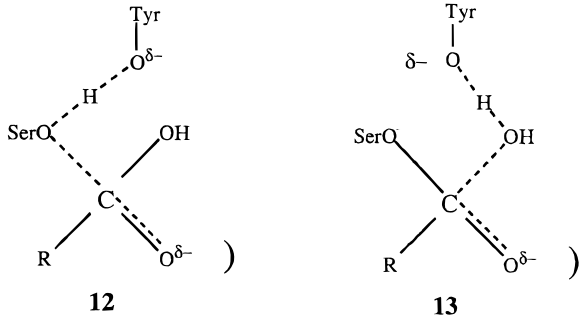
TABLE 3

Interaction Energies (kcal/mol) between the P99 β-Lactamase and Ligands

Complex	E_{int}	E_{Lys}	E_{oxy}
1o	−120.4	−82.7	−63.4
1s	−114.3	−105.4	−65.0
2o	−95.4	−69.3	−57.6
2s	−126.0	−95.1	−58.7
3o	−197.3 (−108.1)[a]	−64.5	−65.3
3s	−244.9 (−131.2)[a]	−100.0	−69.3
15o	−168.4	—	—
15s	−177.0	—	—
16o	−174.0	—	—
16s	−178.1	—	—
4o	−95.5	−41.3	−66.9
4s	−98.0	−71.8	−65.1
5o	−118.2	−41.4	−61.3
5s	−115.5	−55.0	−54.0

[a] After subtraction of the carboxylate contribution.

Another point worth noting at this time with respect to **1** is that for both **1o** and **1s** the minimum energy structure appears to be A rather than B. This result could be interpreted as implying that the transition state **12** is of higher energy than **13** and thus breakdown of the tetrahedral intermediate **1o** via **12** may be rate determining to deacylation rather than its formation via the stabler **13;** the same rationale would also apply to **1s.** This hypothesis could only be confirmed by a full quantum mechanical treatment which was beyond the scope of the present enquiry.



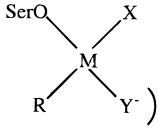
STRUCTURE 4

The E_{int} values for the phosphonates also suggest stronger interaction with the enzyme of the oxo analog **5o** than **5s.** The phosphonate species also prefer structure A (Table 2) and **10o** has previously been interpreted as a mimic of a deacylation transition state *(36,39).* It might be noted in passing here that the thionophosphonate

10s (L = *p*-nitrophenoxide) is a poorer inhibitor of the P99 β-lactamase than **10o** (*46*). The comparison here is of second-order rate constants of irreversible inactivation, however, and presumably reflects the relative stabilities of the pentacoordinated intermediates. No direct comparison with the present structures is therefore possible, although, because of the positioning of the leaving group in phosphonyl transfer reactions (*38*), the pentacoordinated intermediates probably more resemble **5** than **2.** The experimental result is in accord with this conjecture.

More striking than the above are the results for **2** and **3.** These species, containing alkoxide leaving groups at carbon, represent tetrahedral intermediates of the acylation step. The E_{int} values here, however, indicate a significantly stronger interaction of the *thiono* analogs with the enzyme, suggesting preferential thionoester reactivity, contrary to the experimental result. It should be noted here, however, that the lowest energy structure for the oxo species **2o** and **3o** is B whereas for the thiono analogs **2s** and **3s** it is A. This remains true for **3o** and **3s** even when the contributions from carboxylate binding have been subtracted (Table 3), thus indicating that the difference in energy is a property of the tetrahedral MXY^- unit **14.** This structural difference could be interpreted, with the reservations mentioned above with regard to **1o** and **1s,** to indicate that formation of the tetrahedral intermediate may be rate determining to acylation by the thiono esters and breakdown in the case of the oxo, but irrespective of that issue, the calculations suggest that the tetrahedral thiono species formed during the acylation step can interact with the active site residues at least as strongly as the tetrahedral oxo.

Table 3 also shows how two significant contributions to E_{int} vary with structure. One of these, $E_{Lys,}$ the interaction energy between the MXY^- moiety of the ligand (*14*) and the Lys 67 and 315 ammonium ions, has previously been shown to be a significant predictor of the ability of tetrahedral adducts of the P99 β-lactamase to interact with the active site (*36*).



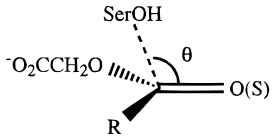
14

STRUCTURE 5

The data of Table 3 show that in all cases **1–5,** MXS^- interacts more strongly with the active site lysines than MXO^-. The difference is balanced by other factors involving internal interactions in E and I in **1** and **5** but not in **2** and **3.** Table 3 also contains $E_{oxy,}$ the energy of interaction between the anion Y^- and the oxyanion hole in each case. As noted previously (*36*), the oxyanion hole interaction does not discriminate significantly between various tetrahedral species, and here not in general between MXS^- and MXO^-. Taken together, the present data are consistent with the previous results and indicate the importance of electrostatic interactions of MXY^- with Lys 67 and Lys 315, but not with the oxyanion hole, in the stronger interaction of thiono than oxo-acylation tetrahedral intermediates **2** and **3** with the active site.

The aldehyde adducts **4** are distinctly different from **1** to **3** and **5,** structurally (O_b replaced by H) and chemically. The latter point has been discussed previously (*36*). The aldehyde adducts represent thermodynamically stable (at sufficiently high aldehyde concentration) but kinetically labile species whereas **1–3** are thermodynamically unstable and kinetically labile and **5** are thermodynamically unstable but kinetically stable. The energy-minimized aldehyde adduct structures **4o** and **4s** are similar but differ between themselves in a different way than **2o** and **2s.** Lysine 73 rather than Tyr 150 (as in structure A) is more closely coordinated with Ser O_γ, particularly in the case of **4o.** Tyr 150 cannot interact with O_b of course, but in **4s,** Lys 315 has come in closer to it and therefore also to Ser O_γ, and to O_a. The upshot of this, energetically, is that E_{int} is larger negative for **4s** than **4o,** probably largely because of the enhanced stabilization of COS^- by Lys 315 (Table 3). On the basis of the calculated E_{int} values, one would expect **4s** to be a more effective (thermodynamic) β-lactamase inhibitor than **4o.** Unfortunately, thioaldehydes such as **4s** are not readily available as stable species under ambient conditions (*47,48*). The effectiveness of **4o** as an inhibitor did, however, increase in HS^- solutions (the apparent K_i decreased from 24 to 10 mM in 10 mM NaHS at pH 7.5; ^1H NMR spectra showed that essentially all of **4o** had been converted into the HS^- adduct under these conditions) but we were unable to trap any thioaldehyde with cyclopentadiene or 1,3-butadiene (*49*) from a stirred biphasic ($H_2O/CHCl_3$) reaction mixture.

The analysis described above suggests that the observed kinetic difference between oxo and thiono substrates arises not because of any steric or electronic difficulty in accommodating tetrahedral thiolates in the active site, but more likely from the presence of unfavorable interactions, or the absence of favorable interactions, very early in the reaction pathway. An attempt was therefore made to assess the relative strength of enzyme-ligand interactions earlier along the reaction coordinate than the first tetrahedral intermediate. In order to do this, a Bürgi-Dunitz trajectory for the reaction was assumed (*42,43*) and the unreacted substrate molecules **8o** and **8s** placed at early positions along it as described under Materials and Methods, viz. **15o(s)** and **16o(s).**



15o (s), θ = 126°
16o (s), θ = 135°

STRUCTURE 6

The energy-minimized structures (not shown) are quite similar in each case. They differ from **3o(s)** in different side chain orientations and in the $-C(=O(S))-OCH_2CO_2^-$ moiety being further out in solution. In particular, the (thiono)carbonyl group is not in the oxyanion hole. The Tyr 150 oxyanion appears in both **15** and **16** to lie within hydrogen-bonding distance of the Lys 73 ammonium ion and Ser O_γ,

the latter as would be expected of a general base catalyst of the acylation reaction (*34,39*). E_{int} values for these species are also presented in Table 3. Noticeable (cf. **3o** and **3s**) is the trend to a smaller difference between the oxo and the thiono analogs as the structure moves back along the reaction coordinate toward reactants and away from the tetrahedral intermediate. Where and whether these exact structures actually occur along the reaction coordinate is not certain, but they agree with experiment in that the strength of noncovalent interactions between the substrates **7o** and **7s** and the enzyme appears to be very similar—dissociation constants of noncovalent complexes were 11 and 5 mM, respectively.

The considerations discussed above appear to suggest that the difficulty that the P99 β-lactamase has in catalyzing the hydrolysis of thiono substrates arises very early in the acylation step, en route to the first tetrahedral intermediate. It is still not clear just what the impediment is, but it must be firmly kinetic in nature, i.e., involving *transitional* species along the reaction coordinate. Consideration of the structural changes needed to convert the energy-minimized structures **15o(s)** and **16o(s)** into **3o(s)** suggested a crank-like motion of the substrate to bring the carbonyl carbon atom into bonding distance with respect to Ser O_γ and the carbonyl oxygen into the oxyanion hole. This would appear to involve, in particular, rotation about the CH_2–CO bond of the side chain and the O–CH_2 bond of the leaving group. The orientation of the side chain amide also changes during this motion which appears to correlate with motion of the Asn 152 side chain. The bulkier thiono carbonyl of **7s** may, for example, have greater difficulty than the oxygen of **7o** in maneuvering past C_β of Ser 318 into the oxyanion hole while maintaining optimal positioning with respect to the catalytic Lys 73/Tyr 150 system.

It would be appropriate to note in passing at this point that the thiono effect might, in principle, be alternatively explained by stabilization of the oxyanion through its formation of a low barrier hydrogen bond (LBHB) with a hydrogen bond donor of the oxyanion hole. This possibility has been suggested by Gerlt and Gassman (*50*) with reference to serine proteinases. It may well be of course that the pK_a of the substrated-derived (protonated) oxyanion better matches the pK_as of the amide hydrogen-bond donors of the oxyanion hole than that of the more acidic thiol analog, and this situation could, according to the LBHB canon, lead to the observed oxygen selectivity. No evidence for an oxyanion LBHB, however, has been claimed from crystal structures of the tetrahedral anionic adducts of serine proteinases and transition state analog inhibitors, e.g., peptidyl aldehydes and ketones (*51–57*), or from ^{1}H NMR studies of such adducts (*58*), although such evidence has perhaps not yet been adequately analyzed from the LBHB perspective. The role of LBHBs in enzymic catalysis is still a controversial issue (*59–65*). LBHBs are not included in the force field employed in the present calculations.

The Class A S. aureus PCl β-Lactamase

The active site of class A β-lactamases is similar to that of the class C enzymes but does differ in significant ways (*33,39,66,67*). In particular, the hydroxyl group of Tyr 150 is replaced by one derived from a serine residue (Ser 130) and a new pair of functional groups is found on the floor of the active site, Glu 166 and Asn 170. Hydrogen-bonded between the side chains of the latter residues is normally found a

water molecule which is believed to be the one involved in hydrolysis of the acyl enzyme, a reaction thought to be catalyzed by the Glu 166 carboxylate acting as a general base (*31*,*33*). Since the S test did appear to indicate the existence of a functional oxyanion hole in a class A β-lactamase *(30)* (the thiono β-lactam **11** was a poor substrate of the TEM β-lactamase; current experiments also showed that **8s** was not a substrate of either the TEM or the *S. aureus* PC1 enzyme), and the crystal structure of a specific phosphonate complex of the class A PC1 β-lactamase of *S. aureus* was available (*44*), the computations described above were performed on PC1 structures analogous to **1** and **2.**

The energy-minimized structures of the PC1 complexes, **1** and **2,** did not greatly differ from the crystal structure except for some changes in ligand side chain orientation similar to those seen in the P99 structures. The oxyanion remained firmly in the oxyanion hole and Lys 73, Ser 130, and Lys 234 maintained their positions with respect to the ligand. The "hydrolytic" water molecule remained firmly embedded between Glu 166, Asn 170, and Ser O_γ. It might be noted, however, that in the **2s** structure (Fig. 3), the Ser 130 hydroxyl oxygen had moved away from the Lys 73 ammonium ion and into hydrogen-bonding distance of the ligand O_b. In the **1o, 1s,** and **2o** (Fig. 3) structures, the Ser 130 hydroxyl remained further away and comparably distanced from Ser 70 O_γ and O_b. It is interesting to note in this context that some suggested acylation mechanisms have employed the Ser 130 hydroxyl group as a general acid to assist departure of the leaving group, in a manner analogous to that suggested for Tyr 150 of the class C enzyme (*34*,*68*,*69*).

The calculated E_{int} values for **1** and **2** (Table 4) are interesting since they both show stronger interaction of the thiono analogs with the enzyme than the oxo. The E_{Lys} and E_{Oxy} values for **1** and **2** indicate, as for the P99 enzyme, that the interactions of the MXY^- moiety with the active site lysines at the tetrahedral intermediate stage of the reaction are not the source of any discrimination against sulfur, quite the reverse in fact, and nor, in general, is the interaction of the oxyanion with the oxyanion hole.

These results therefore indicate that **1s** and **2s** appear to have no more difficulty in residing in the active site than **1o** and **2o,** respectively. The conclusions reached as to the origin of the thiono effect in class C β-lactamases may also obtain in class A.

Chymotrypsin

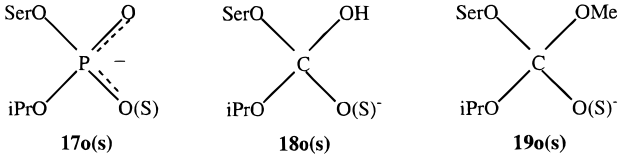
As described in the introduction, the thiono effect was first observed in serine proteinases such as chymotrypsin (*21–23*). It seemed appropriate therefore to briefly at least examine the present method on that system. The crystal structure of the

TABLE 4

Interaction Energies (kcal/mol) between the PC1 β-Lactamase and Ligands

Complex	E_{int}	E_{Lys}	E_{Oxy}
1o	−102.6	−69.6	−74.9
1s	−115.4	−97.2	−76.7
2o	−103.2	−61.1	−71.8
2s	−121.8	−91.8	−63.8

monoisopropylphosphoryl derivative **17o** was used as the starting point (*70*). This was transformed computationally into the tetrahedral carbon species **18o(s)** and **19o(s).**



STRUCTURE 7

Application of the energy-minimization procedures described above to **18o(s)** and **19o(s)** led to final structures of these complexes (not shown) which had changed little from the crystal structure. Asp 102 of the catalytic triad remained firmly hydrogen-bonded to the His 57 cation and the $O(S)^-$ oxyanion remained in the oxyanion hole, although interacting more closely with the backbone NH of Gly 193 than that of Ser 195. The results did present some features in common with those of β-lactamases. First, in terms of structure, in **18o** and **18s,** the deacylation tetrahedral intermediates, His 57 remained, as in the crystal structure, more closely associated with, and presumably hydrogen-bonded to, O_b. In the acylation tetrahedral intermediate structures, however, **19o(s),** and particularly in **19s,** His 57 associated more closely with Ser O_γ than with O_b. The acylation intermediates are of course, as with the β-lactamases, more relevant to the thiono effect.

The catalytically important interaction energies in the energy-minimized complexes are presented in Table 5. The significant result here appears to be that in the carbon-centered species **18** and **19,** the thiono derivatives interact more strongly with His 57 and the oxyanion hole than their oxo analogs. This result suggests that the conclusions made with respect to the genesis of the thiono effect in β-lactamases may well apply to classical serine hydrolases also.

CONCLUSIONS

The thiono effect which has been used empirically to detect the presence of a catalytically active oxyanion hole in serine proteases has been shown to operate also in an acyclic depsipeptide substrate of serine β-lactamases. Although this substrate is not as specific as β-lactams for these enzymes, its additional flexibility did not

TABLE 5

Interaction Energies (kcal/mol) between Active Site Elements of Chymotrypsin and Ligands

Complex	E_{His}	E_{Oxy}
19o	−39.6	−51.9
19s	−44.3	−58.9
20o	−39.4	−51.6
20s	−44.6	−56.3

lead to observable catalysis of its hydrolysis. Discrimination against the thiono substrate occurs during the acylation step. Computer modeling suggests that discrimination does not occur at the tetrahedral intermediate stage of acylation where adducts of the thiono esters appear to interact with the active site at least as strongly as those of the oxo analog. This conclusion appears to hold for both class A and C β-lactamases and also to chymotrypsin, selected as a typical serine protease. The source of the thiono effect must then lie in purely transition state interactions; complete understanding of it may well require a complete quantum mechanical treatment of the acylation reaction.

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