VOLUME 118, NUMBER 32
AUGUST 14, 1996

JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Crystal Structure of 6α-(Hydroxymethyl)penicillanate Complexed to the TEM-1 β-Lactamase from *Escherichia coli*: Evidence on the Mechanism of Action of a Novel Inhibitor Designed by a Computer-Aided Process

Laurent Maveyraud,[†] Irina Massova,[‡] Catherine Birck,[†] Kazuyuki Miyashita,[‡] Jean-Pierre Samama*,[†] and Shahriar Mobashery*,[‡]

Contribution from the Groupe de Cristallographie Biologique, Institut de Pharmacologie et de Biologie Structurale du CNRS, 205 route de Narbonne, 31077 Toulouse cedex, France, and Department of Chemistry, Wayne State University, Detroit, Michigan 48202-3489

Received March 25, 1996[⊗]

Abstract: The crystal structure of the complex of the TEM-1 β-lactamase from *Escherichia coli* inhibited by 6α-(hydroxymethyl)penicillanic acid (**1**) is reported herein. This is the first structure for an acyl-enzyme intermediate with a substrate reported for a native class A β-lactamase. This compound was designed and synthesized as a molecule that would acylate the active site of the enzyme, but would resist deacylation by virtue of the fact that its $C_{6\alpha}$ hydroxymethyl moiety was expected to occupy the space near the hydrolytic water molecule (*J. Am. Chem. Soc.* **1995**, *117*, 11055). The crystal structure of the acyl-enzyme species is closely similar to one of the two energy-minimized acyl-enzyme models generated in the course of the design aspect of the work. The crystal structure provides evidence for a number of mechanistic features for the inhibition process and the ultimate recovery of the activity. Our results reported herein are consistent with the side-chain carboxylate of Glu-166 being the active-site basic function that activates the hydrolytic water for the deacylation step in the course of catalysis by class A β-lactamases. The design principles applied for compound **1** hold the promise of general utility for development of novel inhibitors for other hydrolytic enzymes.

We reported recently on the properties of 6α-(hydroxymethyl)penicillanic acid (**1**) as a novel inhibitor for the class A TEM-1 β-lactamase, which was shown to acylate the enzyme active site, but resisted deacylation.[1] This molecule was designed on the basis of the knowledge of the topology of the enzyme active site and the mechanism of enzyme action. The design process commenced with the energy-minimized structure for the acyl-enzyme intermediate for penicillanic acid (**2**), a reasonably good substrate for the TEM-1 β-lactamase. The 6α-hydroxymethyl group was incorporated into the structure of penicillanate with the intention of displacing the hydrolytic water (Wat-301; referred to as Wat-712 in our previous publication[1]) from the active site upon enzyme acylation by the substrate. Compound **1** proved to be a potent inhibitor for the TEM-1 β-lactamase. The kinetic parameters for both enzyme inhibition and the enzymic turnover of **1** have already been reported.[1] An interesting aspect of the enzyme inhibition profile was the biphasic nature for both the onset of inhibition and recovery of activity from it. This biphasic character of the kinetics was shown not to be associated with a protein conformational change.[1]

[†] Institut de Pharmacologie et de Biologie Structurale du CNRS.
[‡] Wayne State University.
[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1996.
(1) Miyashita, K.; Massova, I.; Taibi, P.; Mobashery, S. *J. Am. Chem. Soc.* **1995**, *117*, 11055.

S0002-7863(96)00971-7 CCC: $12.00

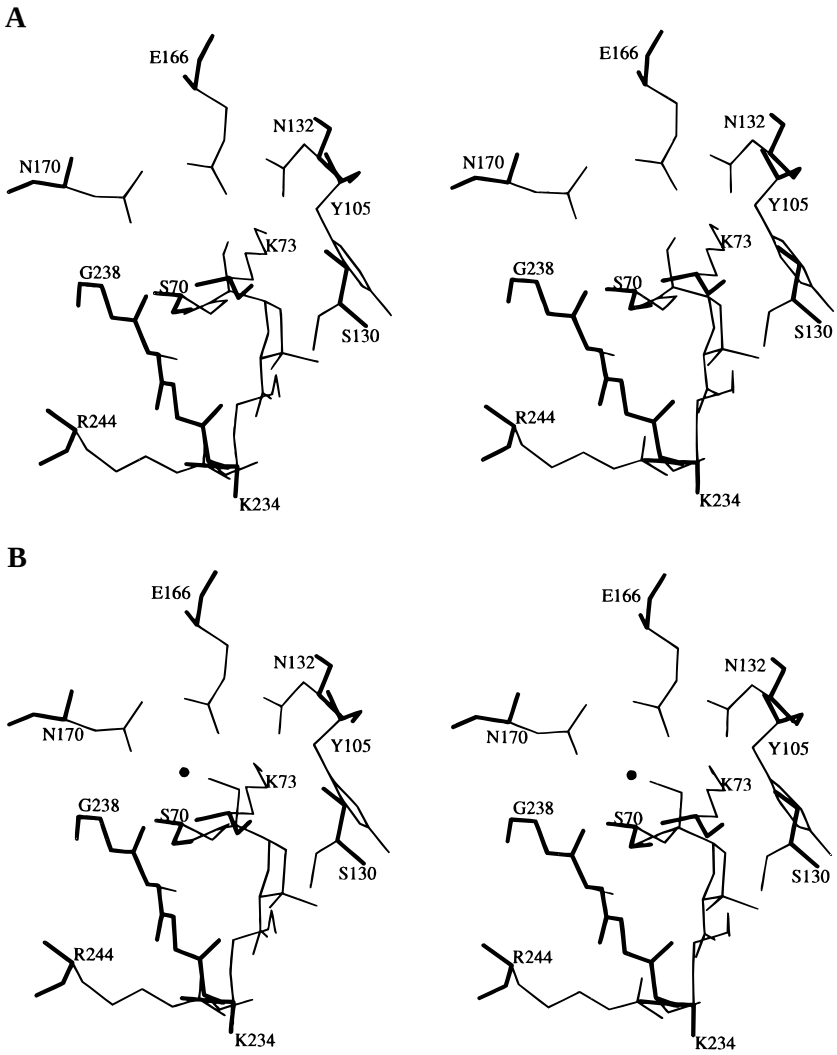
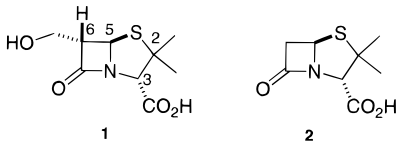


Figure 1. Stereoview of the energy-minimized structures for the acyl-enzyme intermediate of inhibitor **1**. One model (A) showed the displacement of the hydrolytic water, whereas the other (B) retained the hydrolytic water (black sphere), but the hydroxymethyl moiety donated a hydrogen bond to it. The main chain is represented as bold lines.



We will present crystallographic evidence herein that supports the validity of the design paradigms for compound **1**. Furthermore, since this report is the first for a structure of an acyl-enzyme intermediate with a substrate reported for a native class A β-lactamase, the crystallographic findings have implications for the mechanism of turnover of typical substrates by these enzymes. The design principles utilized for compound **1** hold the promise of general utility for development of novel inhibitors for other hydrolytic enzymes.

Experimental Section

The synthesis for compound **1**[1] and the approach for modeling and energy-minimization protocols used in the design process were according to procedures reported earlier.[1−3]

Table 1. Data Processing Statistics, Given for the Entire Resolution Range (22.0−1.95 Å) and for the Highest Resolution Shell (2.02−1.95 Å)

	global	2.02−1.95 Å
no. of reflections	42284	3622
no. of unique reflections	17104	1524
completeness (%)	96.2	94.8
R_{sym} (%)	4.6	7.6
$\langle I/\mathrm{sig}(I)\rangle$	11	8.7

Crystal Preparation and Soaking of the Inhibitor. The TEM-1 β-lactamase crystals were prepared as previously described.[4] Subsequent to crystal growth, medium-sized crystals ($380 \times 200 \times 200\ \mu m^3$) were stabilized by a stepwise increase of the ammonium sulfate content to 50% saturation. Crystals were mounted in Lindeman glass capillaries with a known volume of mother liquor, and a solution of the inhibitor was added for soaking of the crystals. The stock solution of the inhibitor was 250 mM in 50% ammonium sulfate, 100 mM Na,K-phosphate, pH 7.8. Three concentrations of the inhibitor (60, 75, and 85 mM) and three soaking times (150, 210, and 270 min) were employed. The solution was removed from crystals immediately prior to data collection.

Data Collection and Processing. Data were collected on the X31 synchrotron beam line of the EMBL outstation at DESY (Hamburg, Germany), tuned at a wavelength of 0.933 Å. The crystal to detector

(2) Bulychev, A.; Massova, I.; Lerner, S. A.; Mobashery, S. *J. Am. Chem. Soc.* **1995**, *117*, 4797.

(3) Bulychev, A.; O'Brien, M. E.; Massova, I.; Teng, M.; Gibson, T. A.; Miller, M. J.; Mobashery, S. *J. Am. Chem. Soc.* **1995**, *117*, 5938.

(4) Jelsch, C.; Lenfant, F.; Masson, J. M.; Samama, J. P. *J. Mol. Biol.* **1992**, *223*, 377.

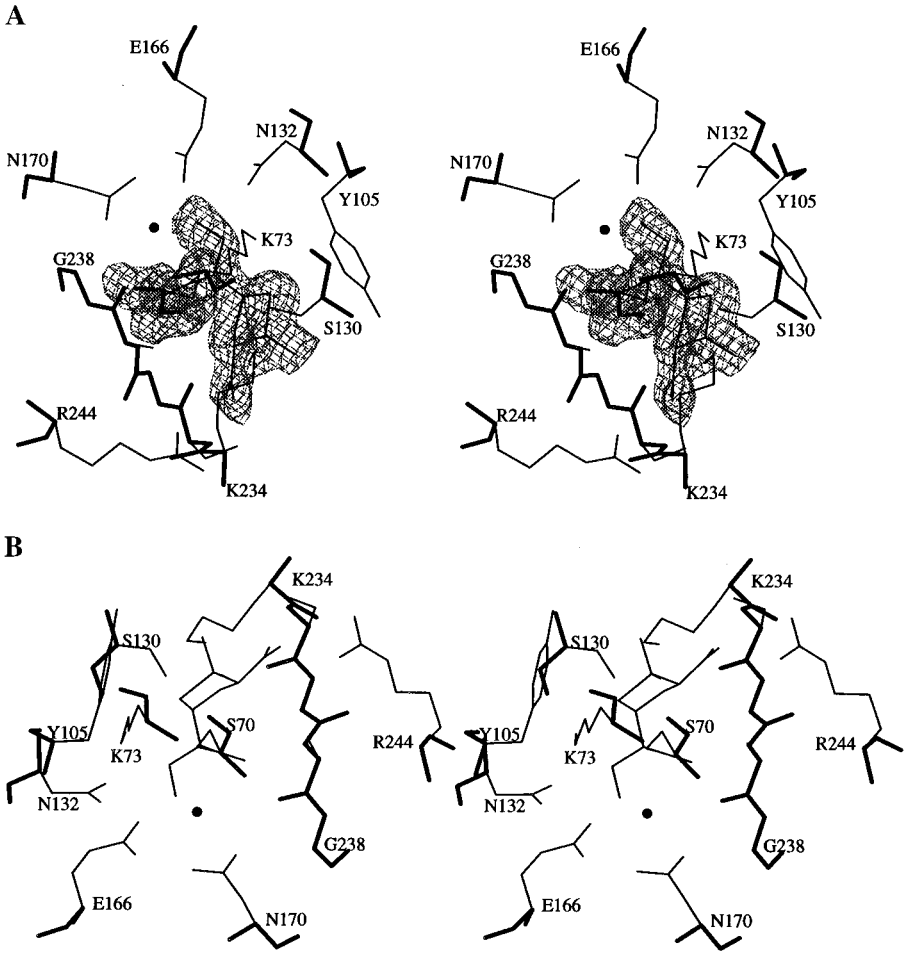


Figure 2. (A) Stereoview of the electron density for the acyl-enzyme intermediate for compound **1**, shown in the same perspective depicted in Figure 1. (B) A different perspective for the crystal structure of the acyl-enzyme complex. The main chain is represented as bold lines. The hydrolytic water (Wat-301) is shown as a black circle. The final $2F_{obs} - F_{calc}$ is shown contoured at 1 standard deviation.

(small MarResearch imaging plate) distance was 170 mm, and the crystal temperature was set to −10 °C. A total of 62 frames (1° oscillation per frame, approximately 4 min exposure time) were collected. Data were processed with MOSFLM[5] and the CCP4[6] packages (Table 1).

Structure Refinement. The structure was refined with the program X-PLOR, version 3.1.[7] A total of 10% of the reflections were randomly selected in the entire dataset in order to provide a test set for the R_{free} calculations.[8] These reflections were not used during refinement, but were included in the electron density map calculations. Models and electron density maps were displayed with the program O.[9]

The 1.8 Å refined TEM-1 β-lactamase structure[10] was used as a starting model for a rigid-body refinement between 7 and 3 Å in order to account for the variations of the crystal cell parameters. This was followed by molecular dynamics refinement in the resolution range 8−1.95 Å, using a slow cooling protocol starting from 3000 K, and two thermal agitation factors per residue. The resulting electron density maps were of good quality and were used to position the inhibitor in the active site. Water molecules were introduced as neutral oxygen atoms when they appeared as positive peaks in the $F_{obs} - F_{calc}$ electron density map contoured at 4 standard deviations above the mean value. Additional refinement cycles (slow cooling protocol at 500 K, energy minimization, and individual thermal factor refinement) and manual corrections to the model were performed to give the final structure described herein.

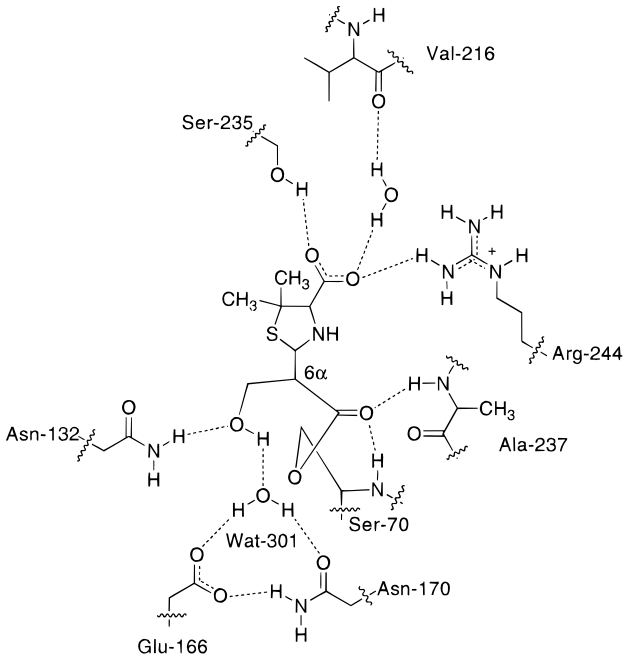
Results and Discussion

The design of compound **1** commenced with the energy-minimized complex for the acyl-enzyme species of penicillanate in the active site of the TEM-1 β-lactamase. Mere introduction of the $C_{6\alpha}$ hydroxymethyl group in penicillanate appeared to be sufficient for bringing the hydroxyl group of this moiety close to the space that the hydrolytic water (Wat-301) occupies in the active site. Two distinct energy minima for the acyl-enzyme intermediate for 6α-(hydroxymethyl)penicillanic acid (**1**) were arrived at. Figure 1A, which was communicated earlier,[1] shows one of these minima, where the hydroxyl of the hydroxymethyl moiety of the inhibitor displaced the hydrolytic water. In the other model (Figure 1B), the hydroxyl of the hydroxymethyl group donated a hydrogen bond to the hydrolytic water. Compound **1** was subsequently synthesized and was shown to be an excellent inhibitor for the TEM-1 β-lactamase.[1]

In order to gain structural information into the mechanism of inhibition, we have determined the crystal structure for the TEM-1 β-lactamase inhibited by compound **1**. The quality of the crystals of the inhibited enzyme, produced by soaking of the inhibitor with crystals of the TEM-1 β-lactamase, was found to be dependent on the duration of the soaking process. The best results were obtained with 75 mM inhibitor and a soaking

(5) Leslie, A. G. W. In *Computational aspects of protein crystal data analysis, Proceedings of the CCP4 Study Weekend*; compiled by Helliwell, J. R., Machin, P. A., Papiz, M. Z.; Daresbury Laboratory, pp 39−50.
(6) Collaborative Computational Project Number 4. *Acta Crystallogr.* **1994**, *D50*, 760.
(7) Brünger, A. T. *X-PLOR manual*, version 3.1; Yale University Press: New Haven and London, 1992.
(8) Brünger, A. T. *Acta Crystallogr.* **1993**, *D49*, 24. Brünger, A. T. *Nature* **1992**, *355*, 472.
(9) Jones, T. A.; Zou, J. Y.; Cowan, S. W.; Kjeldgaard, M. *Acta Crystallogr.* **1991**, *A47*, 110.
(10) Jelsch, C.; Mourey, L.; Masson, J. M.; Samama, J. P. *Proteins: Struct., Funct,, Genet.* **1993**, *16*, 364.

Scheme 1



time of 150 min. Longer duration for soaking led to lack of isomorphism and, ultimately, to cracking of the crystals. These observations may have mechanistic implications, which are discussed later in the text.

Crystals of the complex diffracted to 1.93 Å resolution. They belong to the space group $P2_12_12_1$, but are not isomorphous to the TEM-1 crystals. The cell parameters changed from a = 43.1 Å, b = 64.4 Å, c = 91.2 Å for the free enzyme to a = 42.1 Å, b = 63.1 Å, c = 88.8 Å after the soaking period. Rigid-body refinement, using reflections between 7 and 3 Å, dropped the R_{factor} from 0.451 to 0.327 and the R_{free} from 0.461 to 0.322. The electron density maps, after further refinement between 8 and 1.95 Å, showed most of the features of the inhibitor molecule bound in the active site as an acyl-enzyme complex. The electron density was continuous from Ser-70 O_γ to the carbonyl carbon atom of the inhibitor (Figure 2 and Scheme 1). All protein atoms, except a few solvent-accessible side chains, were visible in the final electron density map. Three alternative side-chain conformations (Glu-58, Ile-260, and Ser-285) were observed, and 234 water molecules were included during the process of structure refinement. The final R_{factor} is 0.156 (R_{free} is 0.202) for all reflections between 8 and 1.95 Å. The evolution of these crystallographic parameters with resolution is given in Figure 3.

The protein structure in the complex is nearly identical to the structure of the unmodified native enzyme. This is supportive of the design expectations, as the inhibitor was designed to fit in the active site of the enzyme on Ser-70 acylation. The root mean square deviation between the main-chain atoms is 0.16 Å after a superimposition of the two structures based on all α-carbon atoms. The maximum root mean square deviation is 1.0 Å for His-96 main-chain atoms. There is no significant difference in the atomic positions of the active-site residues upon complex formation, except for the Ser-130 O_γ. The side chain of Ser-130 is slightly displaced through a small $\chi 1$ dihedral angle rotation, and the hydroxyl oxygen is found at 2.9 Å from the Lys-234 side-chain amino group and at 3.1 Å from the nitrogen atom of the thiazolidine ring of the inhibitor.

The sulfur atom of the thiazolidine ring is at van der Waals distance (3.3 Å) from the Tyr-105 side-chain atoms $C_{\epsilon 1}$ and

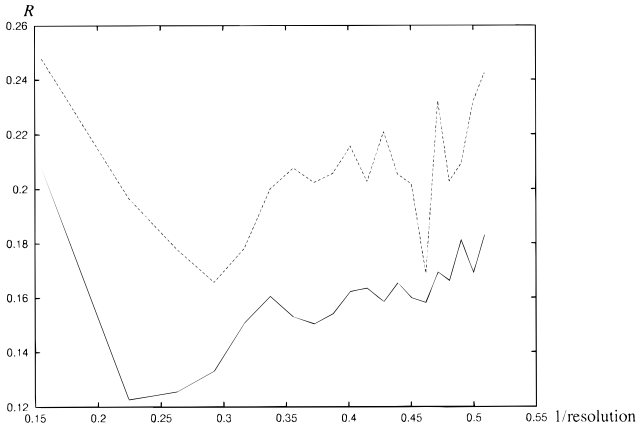


Figure 3. Variation of R_{factor} (solid line) and of R_{free} (dashed line) plotted against the inverse of resolution.

$C_{\delta 1}$. The electron density of this side chain is well defined in the crystal structure of the complex, in contrast to what was observed in the native enzyme structure. The α-methyl group at C_2 of the thiazolidine ring is oriented toward the side chain of Val-216, located at van der Waals contact distance (3.9 Å). The carboxylate substituent at C_3 of the inhibitor is pointing toward the side chain of Arg-244. One of the carboxylate oxygen atoms is at 2.7 Å from Arg-244 $N_{\eta 1}$ and at 2.7 Å from the water molecule bound to the main-chain oxygen atom of residue 216. This water molecule was implicated to play an important role in the mechanism of inhibition of class A β-lactamases by the clinically used mechanism-based inactivator clavulanic acid after formation of the acyl-enzyme complex.[11] The second oxygen atom of the carboxylate group is at 2.7 Å from the Ser-235 O_γ. The involvement of the side-chain functions of residues 244 and 235 in interactions with carboxylates of substrates has been studied previously,[12] and these interactions were seen in the crystal structure for the acyl-enzyme intermediate for the deacylation deficient Glu-166-Asn mutant variant of the TEM β-lactamase with a penicillin substrate.[13] The closest distance between the inhibitor carboxylate and the Lys-234 side-chain ammonium group is 4.6 Å.

The carbonyl oxygen atom of the ester of the complex is located in the oxyanion hole, at equal distances of 2.8 Å from each of the main-chain nitrogen atoms of residues Ala-237 and Ser-70. These hydrogen bonds are nearly in the plane of the peptide bonds of residues 69−70 and 236−237, an arrangement which allows optimal charge transfer effects during active-site acylation.[14]

Glu-166 $O_{\epsilon 2}$ and Asn-170 $N_{\delta 2}$ are at hydrogen-bonding distance (2.9 Å) from one another and do not seem to interact with the oxygen atom of the 6α-hydroxymethyl moiety of the inhibitor located at distances of 3.4 and 3.2 Å, respectively. The two other terminal atoms of the side chains of Glu-166 and of Asn-170 (Glu-166 $O_{\epsilon 1}$ and Asn-170 $O_{\delta 1}$) hydrogen bond with the hydrolytic water molecule (distances of 2.6 and 2.7 Å, respectively), which in turn is located at 3.0 Å from the ester carbonyl carbon of the inhibitor in the complex. This water

(11) Imtiaz, U; Billings, E.; Knox, J. R.; Manavathu, E. K.; Lerner, S. A.; Mobashery, S. *J. Am. Chem. Soc.* **1993**, *115*, 4435. Miyashita, K.; Mobashery, S. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 1043.

(12) Zafaralla, G.; Manavathu, E. K.; Lerner, S. A.; Mobashery, S. *Biochemistry* **1992**, *31*, 3847. Imtiaz, U.; Manavathu, E. K.; Lerner, S. A.; Mobashery, S. *Antimicrob. Agents Chemother*. **1993**, *37*, 2438. Dubus, A.; Wilkin, J. M.; Raquet, X.; Normark, S.; Frère, J. M. *Biochem. J.* **1994**, *301*, 485.

(13) Strynadka, N. C. J.; Adachi, H.; Jensen, E. E.; Johns, K.; Sielecki, A.; Betzel, C; Sutoh, K.; James, M. N. G. *Nature* **1992**, *359*, 700.

(14) Swarén, P.; Maveyraud, L.; Guillet, V.; Masson, J. M.; Mourey, L.; Samama, J. P. *Structure* **1995**, *3*, 603.

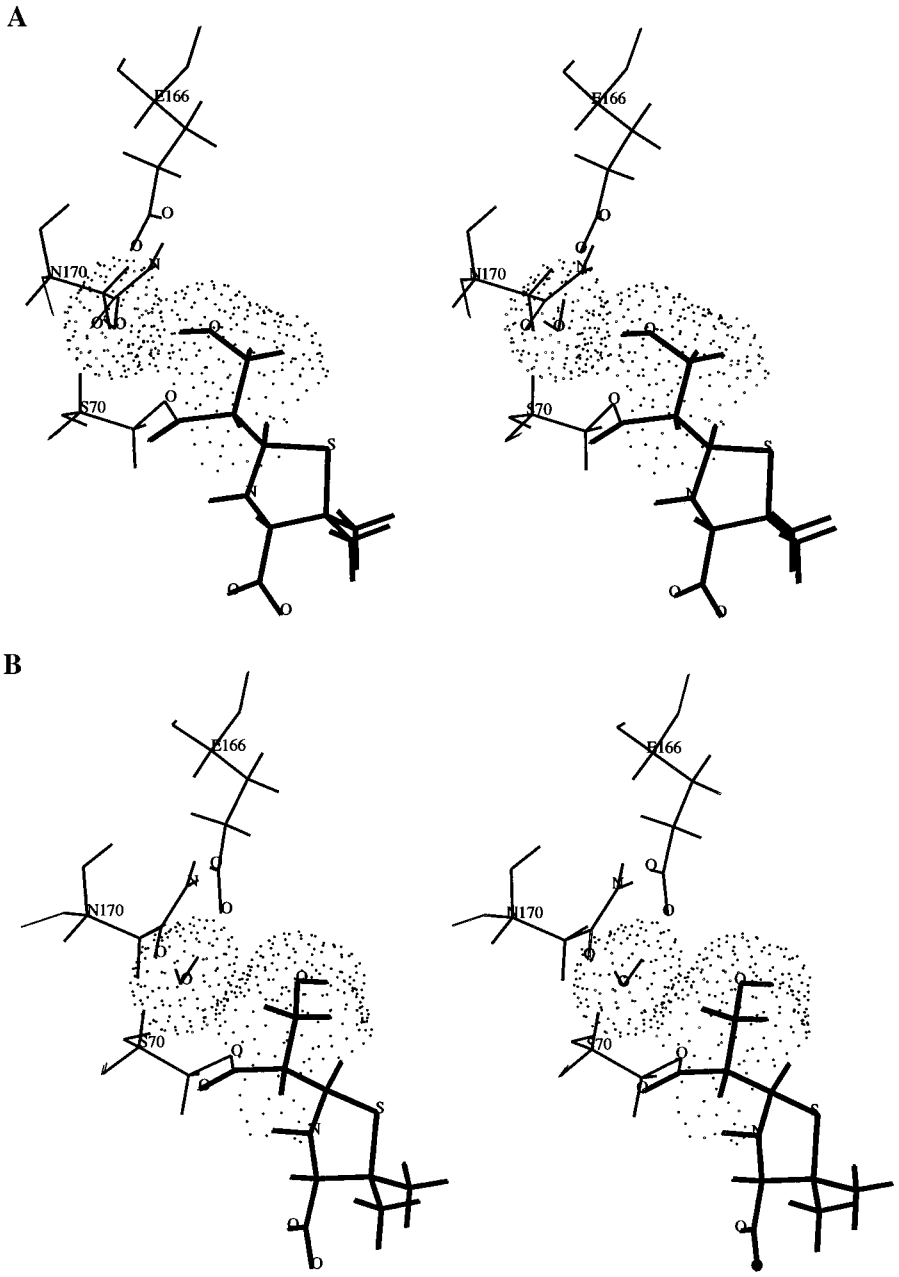
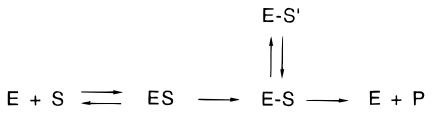


Figure 4. (A) The physical barrier presented by the hydroxymethyl group hydrogen bonded to the hydrolytic water in the crystal structure is shown by the Connolly solvent-accessible surface for the hydroxymethyl group and the hydrolytic water. Hydrogens were added at calculated positions using Sybyl 6.22. (B) The energy-minimized model for the complex after the hydrogen bond between the hydroxymethyl group and the hydrolytic water was broken, showing the lack of steric hindrance for the attack of the hydrolytic water at the ester carbonyl.

molecule also receives a hydrogen bond from the hydroxyl group of the 6α-hydroxymethyl moiety (2.7 Å).

We find it significant that the conformations and interactions in the crystal structure of the acyl-enzyme intermediate for compound **1** are in agreement with those of the energy-minimized computational model shown in Figure 1B. The observations of the details of the crystal structure and the two energy minima are all relevant to the mechanism of action of the enzyme and its inhibition by **1**. As stated earlier, the onset of inhibition and the ultimate recovery of enzyme activity from inhibition are both biphasic processes.[1] Therefore, two distinct binding modes by the inhibitor could account for the difference in the properties of the two inhibited species. We suggest that the inhibited enzyme species shown in Figure 1A, which has displaced the hydrolytic water molecule from the active site, is a likely structure for the inhibited species with the slow onset and a slow rate for recovery of activity. On the other hand, the inhibited enzyme species shown in Figure 1B, and observed in the crystal structure (Figure 2), represents the species with the fast onset and rapid recovery of activity, since the hydrolytic water is still retained. Therefore, we propose that the following minimal scheme explains the inhibition behavior of our inhibitor. After the Michaelis complex (ES) formation, the enzyme gets acylated to give the species E−S. E−S is the complex for the fast phase of inhibition and fast phase for recovery from inhibition. E−S exists in equilibrium with E−S′. E−S′ has to revert back to E−S before hydrolysis takes place, so recovery of activity, by necessity, has to be slower from E−S′ than from E−S. We suggest that this process is at the root of the biphasic nature of the kinetics. *Both E−S and E−S′ would inhibit the enzyme*, but for different reasons. The E−S′ *cannot* directly undergo hydrolysis, since it does not have the hydrolytic water molecule in the active site. After E−S′ reverts back to E−S, the hydrolytic water gets back into the active site, but the hydroxyl of the inhibitor forms a hydrogen bond to it. It is significant to note that the hydroxymethyl moiety at $C_{6\alpha}$ of the

inhibitor presents a physical barrier to the travel of the hydrolytic water toward the ester carbonyl of the acyl-enzyme intermediate. For reasons of steric hindrance for the approach of the hydrolytic water at the ester carbonyl, and also reduced nucleophilicity of the hydrolytic water because of the additional hydrogen bond from the inhibitor to it, species E−S (Figure 2) also inhibits the enzyme.



In order to hydrolyze the ester to permit for the recovery of activity from the inhibited species shown in the crystal structure, it is necessary that the hydrogen bond between the inhibitor hydroxyl and the hydrolytic water molecule be broken. A rotation of the hydroxymethyl group about the bond to C_6 would remove the physical barrier for the travel of the hydrolytic water to the ester carbonyl, but also permits for reversion of the nucleophilicity of the water molecule back to the original state found in the native enzyme. This observation is depicted in Figure 4A, where the van der Waals surfaces for the hydroxymethyl group and the hydrolytic water molecule are shown from the crystal structure. Figure 4B shows the result of the rotation and the feasibility of the deacylation step.

The structural differences between E−S and E−S′ are rather subtle. The root mean square deviation for heavy atoms between the two modeled conformations of the inhibitor, excluding the hydroxyl group for the 6α-hydroxymethyl moiety, is 0.13 Å. The significant difference is for the torsion angle of $C_5-C_6-(C-O)_{sidechain}$, which is −167° for the structure shown in Figure 1B and 76° for that shown in Figure 1A. The total root mean square deviation between the sets of the C_α positions for the two models was merely 0.03 Å. Therefore, we believe that the interconversion of E−S and E−S′ would take place by mere rotation of the hydroxyl group of the inhibitor, potentially accompanied by exchange, strengthening, or weakening of various hydrogen bonds. One hydrogen bond is worth approximately 3 kcal/mol.[15] This means that hydrogen bonds are generally made and lost readily due to translations, rotations, or vibrations in any molecule in solution. Therefore, we believe it plausible that once acylation of Ser-70 takes place, the inhibitor would experience motion within the active site, permitting the interconversion of the two species; in essence, the acyl-enzyme complex is dynamic and not static.

A question that remains unanswered is why protein crystallization affords one acyl-enzyme structure and not the other. Obviously, the structure that is seen is that which forms more readily, in other words, the structure formed during the fast phase for the onset of inhibition. We observed that more prolonged soaking of the crystals of the TEM-1 β-lactamase with the inhibitor resulted in crystal cracking. This observation is possibly indicative of the fact that the conversion of the first inhibitory species (E−S) to the other (E−S′) requires local motion of the acyl-enzyme inhibitor, which results in the cracking of the crystals. Similar results were also seen for β-lactamase inhibited by clavulanate. Herzberg and colleagues showed that two of the three species that had been demonstrated to exist in mechanistic studies were not seen in the crystal structure of β-lactamase modified in the active site by clavulanate.[16]

Acknowledgment. The work in France was funded in part by INSERM (CRE Contract 930612; J.P.S.) and the Regional Midi-Pyrenees (Contract 9200843; J.P.S.). The work in the U.S.A. was supported by a grant from the National Institutes of Health (S.M.). I.M. is the recipient of the Rumble and Predoctoral Fellowships.

Supporting Information Available: The crystallographic coordinates have been deposited in the Brookhaven Protein Data Bank.

JA9609718

(15) Olovsson, I.; Jönsson, P. G. In *The Hydrogen Bond*; Schuster, P., Zundel, G., Sandorfy, C., Eds.; North-Holland: Amsterdam, 1976; Vol. 2, pp 393−456.

(16) Chen, C. C. H.; Herzberg, O. *J. Mol. Biol.* **1992**, *224*, 1103.