# Mechanism of Turnover of Imipenem by the TEM β-Lactamase Revisited

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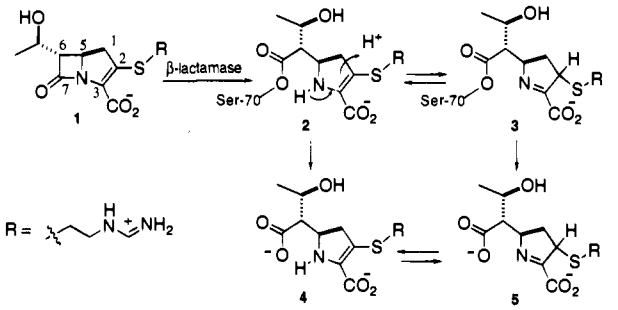
**Abstract:** The mechanism of turnover of imipenem, a clinically important carbapenem antibiotic, by the TEM-1 β-lactamase has been investigated. The details of the turnover process appear to be more complicated than previously believed. The details of the mechanism of turnover have been explored by the study of energy-minimized structures for the two acyl-enzyme intermediates, for the two enzyme-product complexes, as well as kinetic experiments. Evidence is presented that the unusual stability of the enzyme−product complexes and a subtle protein conformational change in the course of turnover may play roles in the enzymic hydrolysis of imipenem. Furthermore, the rate of deacylation is the slow step in turnover. The slow rate of turnover has been explained by the function of the $C_{6\alpha}$ hydroxyethyl group, which binds in a small pocket in the active site. The hydroxyl of the hydroxyethyl moiety makes a strong hydrogen bond to the side chain of Asn-132, whereby the methyl group of this moiety displaces the hydrolytic water molecule (*i.e.*, Wat-712) by 2−3 Å from its optimal position in the active site. This structural factor has been offered as the basis for turnover of imipenem being as much as $10^3$- to $10^4$-fold slower than with the better substrates for class A β-lactamases.

Carbapenem antibiotics constitute a family of potent broad-spectrum β-lactam antibacterials, a member of which, imipenem, has found clinical utility. Imipenem is generally considered resistant to the action of class A and C β-lactamases,[1] bacterial enzymes which hydrolyze β-lactam drugs as a defensive strategy against these agents. Imipenem retains its activity in the face of β-lactamase-mediated resistance to all other β-lactams; however, in clinical isolates certain variants of the zinc-dependent class B β-lactamases have been identified which hydrolyze carbapenems well.[2] More recently, a class A β-lactamase from *Serratia marcescens* has been shown to turn over imipenem efficiently as well.[3] Carbapenems generally acylate the active site of β-lactamases readily but undergo deacylation sluggishly;[4] as a consequence, they often inhibit β-lactamases. The process of turnover for carbapenems is complicated with kinetics that are biphasic. Knowles and colleagues suggested that the biphasic nature of the kinetics of turnover is due to a tautomerization of the carbapenem nucleus after acylation of the enzyme active site by the compound (Scheme 1).[5,6] We recently reported our preliminary results in support of this mechanism for the turnover of imipenem (**1**), where we suggested that a water molecule coordinated to the Arg-244 side chain may facilitate the tautomerization process by transfer of a proton.[7]

**Scheme 1**




We present here a more detailed structure-based analysis of the imipenem interactions with the active site of the TEM-1 β-lactamase, a class A enzyme, and provide additional insights into the mechanism of inhibition of these enzymes in the process of turnover of imipenem. Such detailed mechanistic understanding is needed in light of the fact that imipenem is considered a "β-lactam of the last resort" in the treatment of multidrug-resistant infections. Furthermore, certain features unique to turnover of imipenem, as disclosed here, may prove helpful in rational design of future generations of β-lactam antibiotics.

## Experimental Section

Amino acid substitution at position 244 of the TEM-1 β-lactamase was carried out by site-specific mutagenesis according to the method of Kunkel,[8] as reported previously,[9] and the enzyme purification protocol for the wild-type and Ser-244 mutant proteins has been described.[9] Benzylpenicillin and imipenem were obtained from Sigma Chemical Co. and Merck and Co., respectively. Kinetic measurements were carried out in a Hewlett-Packard 452 diodearray instrument. Hydrogen- and carbon-NMR spectra were obtained in a Nicolet QE-300 spectrometer at 300 and 75 MHz, respectively. Chemical shift values (δ) are given in ppm. Melting points were taken in a Hoover

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UniMelt apparatus and are uncorrected. Circular-dichroic spectra were recorded on a Jasco Model J-600 spectropolarimeter.

**Modeling.** Crystal coordinates for the TEM-1 β-lactamase[10] were generously communicated by Professor Michael James and were used in the three-dimensional modeling and energy minimization. The crystal structure of imipenem[11] was docked into the active site to permit the formation of the two acyl-enzyme intermediates with the Ser-70 active site. A total of 226 crystallographic water molecules were retained, and the active-site-bound substrates were then individually capped by the addition of 23 Monte-Carlo water molecules in each case. The hydrogen atoms were added in the calculated positions and atomic charges were computed by the method of Gasteiger–Hückel.[12] The energy minimization was performed by the geometry optimization algorithm MAXMIN2, using the Tripos force field by the Sybyl molecular modeling software in a Silicon Graphics R4000 Indigo computer. The Powell method was used to determine the descending direction in the minimization.[13] Energy minimization for each acyl-enzyme intermediate was performed in three stages: (1) the substrate and water molecules were allowed to move; (2) the substrate, water molecules, and the protein backbone were allowed to move; and (3) finally, the entire acyl-enzyme complex was allowed to minimize without any constraints. The minimization in a radius of 15 Å from the active site in each stage was continued until the change in energy was less than 0.001 kcal $mol^{-1}$ $Å^{-1}$ between iterations. A dielectric constant of 1.0 was used for the calculations.

**Profile of Imipenem Hydrolysis and Steady-State Rate Measurements.** The hydrolytic cleavage of the imipenem occurs with a substantial decrease in the absorption band at 300 nm. A solution of imipenem (40 μM final concentration) was incubated with the wild-type TEM-1 β-lactamase (2 μM final concentration) in 1.0 mL of 100 mM sodium phosphate, pH 7.0, at room temperature. The decrease in absorbance at 300 nm was monitored until all of the imipenem had been hydrolyzed, as shown by further lack of change in the absorbance measurement. The change in the extinction coefficient was calculated from the extinction coefficient of the intact imipenem and that of imipenem after complete enzymatic hydrolysis ($\Delta\epsilon_{300}$ = 3550 $M^{-1}$ $cm^{-1}$).

For the monitoring of hydrolysis the method of Charnas and Knowles was followed.[6] The wild-type enzyme solution (2 μM final concentration) was mixed with imipenem (40 μM) in 1.0 mL of 100 mM potassium phosphate, pH 7.0. The progress of the reaction was followed at 300 nm at room temperature. A similar experiment was carried out for the Ser-244 mutant β-lactamase.

The values of $k_{cat}$ and $K_m$ were determined from Lineweaver–Burk plots of the initial steady-state velocities at various imipenem concentrations (20–50 μM) with the Ser-244 enzyme. The spectrophotometric assay of β-lactamase activity was carried out in 50 mM sodium phosphate, 100 mM NaCl, pH 7.0, and the reaction was monitored at 300 nm. Initial rates were determined from the first 5–10% of the reactions with six substrate concentrations bracketing the $K_m$. Each assay of enzymic activity for imipenem was carried out in triplicate.

**Determination of Dissociation Constants.** The dissociation constants ($K_s$) for imipenem with the wild-type and Ser-244 β-lactamase were determined according to the procedure of Zafaralla *et al.*[9] Two concentrations of substrate ([2-[[*p*-(dimethylamino)phenyl]azo]pyridino]-cephalosporin, PADAC; $\Delta\epsilon_{610}$ = 1240 $M^{-1}$ $cm^{-1}$) in 100 mM phosphate buffer, pH 7.0—400 and 800 μM with the wild-type enzyme, and 320 and 400 μM with the Ser-244 β-lactamase—were used in each case. A series of mixtures containing both the substrate and various concentrations of imipenem (0–120 μM for the wild-type and 0–1.1 mM for the Ser-244 enzyme) were prepared in 100 mM phosphate buffer, pH 7.0, in a total volume of 0.5 mL. A portion of the enzyme (final enzyme concentrations of 14 nM for the wild-type and 150 nM for Ser-244 mutant β-lactamase) was added to start the inhibition assays.

The dissociation constants for the hydrolytic products ($K_{i,p}$) of benzylpenicillin and imipenem for β-lactamase were determined by the method of Dixon.[14] These determinations were made with the wild-type β-lactamase for benzylpenicilloate and with both the wild-type and Ser-244 β-lactamase for the imipenem products. For these determinations, 350 and 700 μM concentrations of PADAC in 100 mM phosphate buffer, pH 7.0, were used. A series of mixtures containing both the substrate and various concentrations of benzylpenicilloate (0–33 mM) or the imipenem products (0–3.3 mM for the Ser-244 β-lactamase, and 0–0.7 mM for the wild-type enzyme) were prepared in 100 mM phosphate buffer, pH 7.0. A portion of the enzyme was added to obtain a final concentration of 20 nM for the wild-type and 260 nM for Ser-244 in a total volume of 0.3 mL. The rate of hydrolysis was monitored at 610 nm, as described for the $K_s$ determinations.

**Measurements of the Rate of Imipenem Displacement from the Active Site.** The method of Koerber and Fink was followed.[15] A 250-μL solution of the wild-type β-lactamase (2 μM) and imipenem (20 μM) in 100 mM sodium phosphate, pH 7.0, was incubated at room temperature for 1 min. A 10-μL portion of the mixture was added to a cuvette containing benzylpenicillin (2.0 mM; 990 μL), and its hydrolysis was followed at 240 nm for 15 min. The first-order rate constant for the generation of enzymic activity was determined from the progress curve of benzylpenicillin hydrolysis according to the method of Glick *et al.*[16]

**Synthesis of the Hydrolytic Products of Imipenem.** A modification of the method of Ratcliffe *et al.*[11] was followed. A solution of imipenem (0.5 mg) in 0.05 M aqueous $H_2SO_4$ (1.0 mL) was stirred at room temperature for 1 h. The acidic solution was brought to pH 7.0 by the gradual addition of solid barium hydroxide. The resultant precipitate of barium sulfate was removed by filtration. The product was subsequently purified by HPLC; $t_R$ = 12.6 min (Vydac, $C_{18}$ column, 25 × 1.0 cm, 5 μm particle size, linear gradient 2–98% acetonitrile in water over 30 min, 1.0 mL/min, 215 nm). The product was an equal mixture of $\Delta^1$- and $\Delta^2$-tautomers, as determined from the $^1$H-NMR spectrum. The spectroscopic characteristics of the product mixture were identical to those described by Ratcliffe *et al.*[11]

**Synthesis of the Hydrolytic Product of Benzylpenicillin.** A solution of 200 mg of benzylpenicillin in 10 mM phosphate buffer, pH 7.0 (4 mL), was incubated with the TEM-1 β-lactamase ($1.2 \times 10^{-7}$ M final concentration) at room temperature for 2 h. The mixture was subsequently filtrated through an Amicon YM-10 membrane to remove the enzyme. The hydrolytic product in the filtrate was purify by HPLC; $t_R$ = 13.0 min (Vydac, $C_{18}$ column, 25 × 1.0 cm, 5 μm particle size, linear gradient 2–98% acetonitrile in water over 30 min, 1.0 mL/min, 215 nm). mp ~120 °C (dec); IR (KBr) 1566, 1574, 1655 $cm^{-1}$; $^1$H-NMR ($D_2O$) δ 0.99 (s, 3H, methyl), 1.53 (s, 3H, methyl), 3.37 (s, 1H, $C_3$ methine), 3.76 (s, 2H, side-chain methylene), 4.75 (d, 1H, $C_5$, $J$ = 3 Hz), 5.02 (d, 1H, $C_6$, $J$ = 3 Hz), 7.4 (m, 5H, aromatic); $^{13}$C NMR ($CDCl_3$): δ 27.9 ($CH_3$), 27.7 ($CH_3$), 42.4 (side-chain $CH_2$), 55.1 ($C_3$), 58.4 ($C_2$), 66.9 ($C_5$), 75.8 ($C_6$), 127.5, 129.2, 129.6, 134.5 (aromatics), 174.8, 174.9, 175.2 (carbonyl; two carboxylates and one amide); MS FAB$^+$ 375 (M + 2H, 2%).

**Circular Dichroic Measurements.** Protein concentrations of 7 μM for the wild-type β-lactamase and 70 μM for imipenem, or 300 μM product(s) of imipenem hydrolysis, in 10 mM sodium phosphate, pH 7.0, were used in these experiments. A CD cell with the path length of 1 mm was used in the measurements.

## Results and Discussion

Carbapenems acylate the active-site serine in class A β-lactamases readily, but deacylation takes place more slowly (Scheme 1). The tautomerization of the double bond in the first acylated species (**2** → **3**) is believed to attenuate the rate of deacylation, thereby resulting in a biphasic turnover profile. During the first phase 2–3 molecules of carbapenem are turned over before the onset of the slower phase of hydrolysis. We

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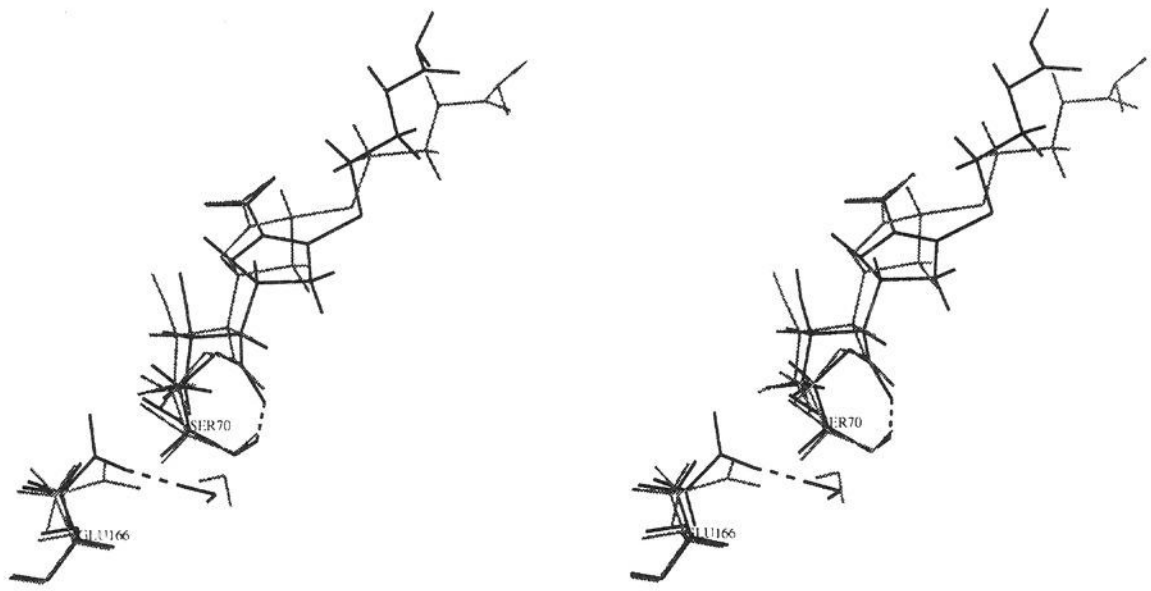


**Figure 1.** The structures for the energy-minimized acyl-enzyme intermediates for the $\Delta^2$- (black) and $\Delta^1$-pyrroline (gray) tautomers. Amino-acid residues Ser-70 and Glu-166 are shown. The water molecule coordinated to the carboxylate of Glu-166 is the displaced hydrolytic water, Wat-712.

had suggested earlier that Arg-244,[17] or a water molecule coordinated to its side-chain guanidinium ion, may be the source of a necessary proton for the tautomerization reaction (Scheme 1).[7] At the time, the assertion regarding the existence of a water molecule coordinated to the Arg-244 side chain was merely a conjecture, since the available crystal structure was devoid of any crystallographic water molecules. Our new models reveal that the likely source of the proton is indeed the structurally conserved Wat-673. This water molecule is chelated in the protein by the side chain of Arg-244 and the main-chain carbonyl of Val-216. This is reminiscent of the model that we proposed recently for the acyl-enzyme intermediate of clavulanate, a mechanism-based inactivator for class A $\beta$-lactamase.[18] We had suggested that Wat-673 serves as the source of a critical proton in the inactivation chemistry of clavulanate. This role for Wat-673 is analogous in the imipenem chemistry. The proton would be introduced from the $\beta$-face of the molecule to result in an *R* configuration for $C_2$ in the $\Delta^1$-tautomer (**3**), although once the product is released, scrambling of the stereogenicity has been shown to take place at neutral pH.[5]

We prepared the Ser-244 mutant TEM $\beta$-lactamase to test the role for Wat-673 in turnover of imipenem.[19] The profile for turnover of imipenem with this mutant enzyme simplified to monophasic kinetics, suggesting that the tautomerization process was avoided for the enzyme-bound species. Because of the simplified kinetics, we were able to evaluate the steady-state turnover kinetics for imipenem with this enzyme ($K_m$ = 27 $\mu$M and $k_{cat} = 0.04\ s^{-1}$).[7] We were able to measure the rate of recovery of activity in the late portions of the second phase of turnover by the wild-type TEM-1 $\beta$-lactamase at $3.3 \times 10^{-3}$ $s^{-1}$. The rate of recovery for neither the Ser-244 mutant enzyme nor the fast phase of the wild-type could be measured accurately, since these rates were too fast for the limit of the technique. If $k_{cat}$ for the Ser-244 mutant enzyme were the lower limit for the recovery of activity, we suggested that a minimum of 12-fold rate difference exists for the recovery of activity between the two phases for the wild-type enzyme.[7] Monk and Waley proposed a branched pathway for turnover of imipenem by the related class A $\beta$-lactamase from *Bacillus cereus* as well,[20] although their scheme was somewhat simpler than that offered by Knowles and colleagues. Monk and Waley estimated that the rate of deacylation ($k_3$) was approximately 6- to 12-fold slower than the rate of acylation ($k_2$) for the *B. cereus* enzyme. If the same rate difference for $k_2$ and $k_3$ were to exist for the related TEM-1 $\beta$-lactamase, the value of $k_{cat}$ (0.04 $s^{-1}$) would be nearly equal to $k_3$, making the deacylation step rate-limiting (*vide infra*) for this enzyme as well. We add parenthetically that the value of $k_{cat}$ for imipenem determined recently for another class A $\beta$-lactamase from *Streptomyces albus* G was identical to the value reported by us for the Ser-244 mutant TEM $\beta$-lactamase (*i.e.*, 0.04 $s^{-1}$).[21]

In our earlier work with imipenem[7] we had not attempted any energy minimization of the acyl-enzyme intermediates since we had access to the crystal structure of a $\beta$-lactamase which did not have the waters of hydration. With the availability of high-resolution crystal coordinates for the TEM-1 $\beta$-lactamase,[10,22] we generated the structures for the $\Delta^2$- and $\Delta^1$-tautomers of the acyl-enzyme intermediate of imipenem (**2** and **3**, respectively) in the active site, and the energies of the complexes were minimized. As depicted in Figure 1, the remarkable similarity of the conformations of the two tautomers for the acyl-enzyme intermediates is striking. The $\Delta^1$-tautomer has a somewhat more puckered structure and its five-membered ring has translated toward Arg-244 (not shown) by less than 0.5 Å. The principal difference between the two structures is that the $\Delta^1$-tautomer allows for a set of bifurcated hydrogen bonds between the side-chain hydroxyl of Ser-130, and the imine nitrogen and the Ser-70 $\beta$-oxygen which is acylated by the substrate; for the $\Delta^2$-tautomer only hydrogen bonding by Ser-130 side chain to the Ser-70 $\beta$-oxygen is seen (data not shown). This differential interaction may be a factor in potential stabilization of the $\Delta^1$- over the $\Delta^2$-acyl-enzyme intermediate. We find it remarkable, however, that the binding at the "oxyanion hole" remains identical in both acylated species (Figure 1). The acyl carbonyl oxygen makes strong hydrogen bonds to the backbone amine of Ser-70 (shown) and Ala-237 (not shown) in each intermediate. This interaction is believed to be important in polarization of the ester carbonyl for the subsequent hydrolytic step.[10,22-24]

(17) The consensus amino acid numbering system of class A $\beta$-lactamases has been used throughout the manuscript (Ambler, R. P.; Coulson, A. F. W.; Frère, J. M.; Ghuysen, J. M.; Joris, B.; Forsman, M.; Levesque, R. C.; Tiraby, G.; Waley, S. G. *Biochem. J.* **1991**, *276*, 269). The water molecules in the crystal coordinates for the TEM-1 $\beta$-lactamase which were made available to us were not numbered. Hence, the numbering system for the water molecules is that of the related *Bacillus licheniformis* enzyme, as proposed by Knox and Moews (Knox, J. R.; Moews, P. C. *J. Mol. Biol.* **1991**, 220, 435).

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(19) The CD spectra of the wild-type TEM-1 and the Ser-244 mutant $\beta$-lactamases were superimposable (see ref 9), indicating a lack of change in the collective secondary structural elements of the protein after mutagenesis.

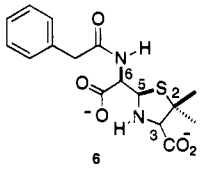
(20) Monk, J.; Waley, S. G. *Biochem. J.* **1988**, *253*, 323.

(21) Matagne, A.; Lamotte-Brasseur, J.; Frère, J. M. *Eur. J. Biochem.* **1993**, *217*, 61.

(22) Jelsch, C.; Mourey, L.; Masson, J. M.; Samama, J. P. *Proteins* **1993**, *16*, 364.

The striking conformational similarity of the two acyl-enzyme intermediates was unexpected. We had initially envisioned that due to the differences in the pucker of the five-membered rings in structures **2** and **3**, the $\Delta^1$-tautomer (*i.e.*, **3**) would provide a physical barrier to the travel of the hydrolytic water, thereby retarding the rate of deacylation of this tautomer. The computational model of Figure 1 is inconsistent with this possibility. We conclude that the sequence of events depicted in Scheme 1 may not adequately describe the process of turnover of imipenem. Two additional possibilities should be considered: (1) the biphasic kinetics may potentially be due to product inhibition—that is, the difference in the rates of release of the two products from the active site may account for the biphasic kinetics; and/or (2) a protein conformational change may discriminate between the two tautomers, either at the acyl-enzyme intermediate or at the enzyme−product complex stages of turnover.

Product inhibition is unprecedented for β-lactamases. However, the computational model indicates that the α orientation of the hydroxyethyl group at the $C_6$ of imipenem allows for favorable interactions with the active site of the enzyme. Specifically, the hydroxyl of the hydroxyethyl moiety makes a hydrogen bond to the side chain of Asn-132, positioning the methyl group into a cavity at the bottom of the active site. Most substrates for β-lactamases have a β orientation at $C_6$ of penicillins or $C_7$ of cephalosporins. This arrangement pushes the product carboxylate—formerly the lactam carbonyl—toward Glu-166 carboxylate. The electrostatic repulsion of these carboxylates should facilitate the ready dissociation of the enzyme−product complex in turnover of most typical substrates. However, for imipenem, this repulsive force has been minimized by positioning of the product carboxylate away from that of Glu-166, dictated by the hydroxyethyl group and its interaction with the active site. In this light, we considered the possibility of a potential product inhibition during turnover of imipenem.[25] We synthesized the products of imipenem hydrolysis as an equal mixture of the $\Delta^1$- and $\Delta^2$-tautomers (**5** and **4**; **5** is comprised of a 1:1 mixture of the two isomers at its $C_2$); the tautomers could not be separated by HPLC. This mixture of products inhibited the TEM-1 β-lactamase in a competitive manner with a $K_{i,p} = 270 \pm 26$ μM. Importantly, after incubation of the enzyme for 20 min in the presence of the mixture of **4** and **5** (1.0 mM), there was an immediate recovery of activity on addition of the incubation mixture to a solution of benzylpenicillin. Hence, we conclude that *the rate of deacylation must be slower than the rate of dissociation of the enzyme−product complex*. In contrast, the product of hydrolysis of benzylpenicillin (**6**) inhibited the enzyme very poorly with $K_{i,p} = 29 \pm 1$ mM. The large difference in affinities of the mixture of **4** and **5**, and **6** for β-lactamase supports our view on the favorable interactions of the 6α-hydroxyethyl moiety with the active site. We note that the Ser-244 enzyme is inhibited somewhat less by the product mixture of imipenem—$K_{i,p} = 1.1 \pm 0.1$ mM—consistent with the view that Arg-244 of the wild-type β-lactamase makes a weak hydrogen bond to the substrate carboxylate in the course of turnover,[7,9,10,18] and as shown here, with the $C_4$ carboxylate of the imipenem product as well.



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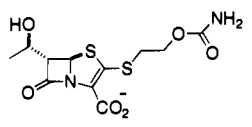
At first glance, the $K_{i,p}$ for the products of imipenem hydrolysis is not impressive (*i.e.*, 270 μM), but it merely argues for a relative stability of the enzyme−product complex(s) in turnover of imipenem. One should bear in mind that imipenem is turned over ($k_{cat}/K_m = 1.5 \times 10^3$ $M^{-1}$ $s^{-1}$ for the Ser-244 β-lactamase), although sluggishly. Hence, the enzyme−product complexes for imipenem, once formed, are apt to exist longer than would the enzyme−product complex for benzylpenicillin, whose dissociation is limited only by diffusion.[26] We hasten to add here that in a previous work on the mechanism of turnover of the penem **7**, we had discovered that the immediate



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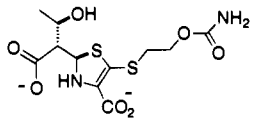

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product of turnover (**8**) was capable of binding the active site of the TEM-1 β-lactamase and be converted to a different compound in a new enzyme-mediated process.[27] Whereas the details of this enzymic conversion are not fully understood at the present, the fact that **8** would bind the active site of β-lactamase with reasonable affinity is in good agreement with the findings disclosed here for the relative stability of the enzyme−product complex(es) for imipenem.

Since turnover of imipenem by the TEM-1 β-lactamase was slow, we were able to investigate by circular-dichroic measurements whether any conformational changes were associated with the protein in the course of turnover. As shown in Figure 2, parts A and B, a subtle change in the far-UV CD—an enhancement of helicity—is seen when imipenem is incubated with the wild-type TEM-1 β-lactamase. The CD spectrum returns back to that of the native protein after the substrate is fully hydrolyzed. The CD spectrum of the Ser-244 β-lactamase shows the same conformational change, except that the extent of the effect on the CD spectrum is somewhat less than that for the wild-type β-lactamase and the length of recovery to the native form takes longer (Figure 2C). The slower recovery to the native CD spectrum shown by the Ser-244 enzyme is consistent with the contribution of a hydrogen bond by Arg-244 to substrate species in the course of turnover. Interestingly, there was no indication for a conformational change in the CD spectrum of the β-lactamase upon incubation of the product(s) of imipenem hydrolysis with the protein as a function of time (data not shown). Conformational elasticity of β-lactamases has been reported in the literature, although direct evidence has been presented only with certain types of substrates[28] or inactivators[29] with unusual hydrophobic side-chain acyl func-

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(25) Modeling was helpful in these analyses. We noted that the enzyme and product(s) gave "energy-minimized" complexes. No distortion of the active site resulted on such energy minimization. The two products appeared to form favorable non-covalent complexes with the active site of the TEM-1 β-lactamase. The $C_7$ carboxylate—the former β-lactam carbonyl—of the $\Delta^1$-product received the maximum full complement of five hydrogen bonds from the protein, which should impart substantial stabilization to the complex. In a qualitative way, the complex of the $\Delta^1$-product appeared somewhat more stable than that of the $\Delta^2$-product from the multitude of interactions with the active site (data not shown).

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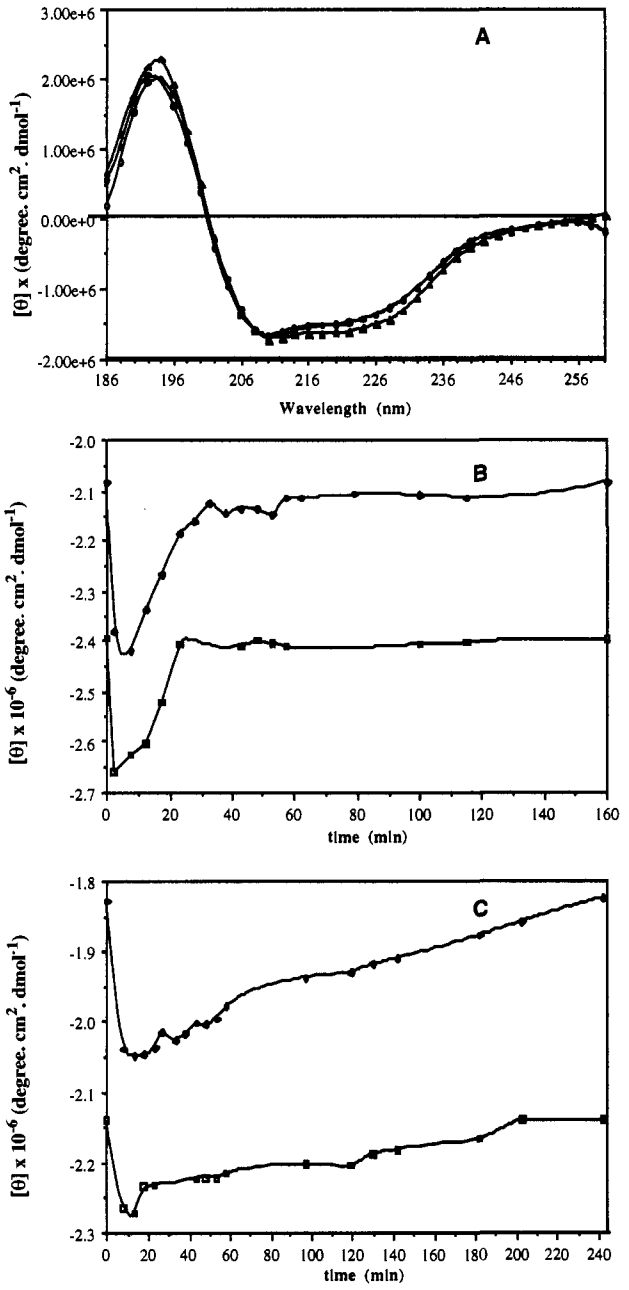
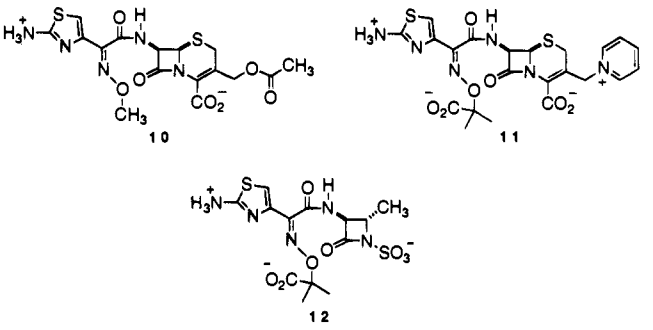



**Figure 2.** (A) The far-UV CD spectrum of the TEM-1 $\beta$-lactamase (○), $\beta$-lactamase during imipenem turnover (▲), and lactamase at the end of hydrolysis of imipenem (●). (B) Change in the molar ellipticity of the wild-type TEM-1 $\beta$-lactamase at 208 (□) and 222 nm (●) as a function of time during the turnover of imipenem. (C) Change in the molar ellipticity of the Ser-244 mutant $\beta$-lactamase at 208 (□) and 222 nm (●) as a function of time during the turnover of imipenem.

tions at $C_{6\beta}$. These moieties presumably interact with the protein to induce a conformational change that retards deacylation. The structural basis for such interactions with $\beta$-lactamases has not been investigated; however, because of substantial structural differences, the nature of these interactions has to be different from those discussed here for imipenem. To our knowledge, imipenem is the only compound without the unusual hydrophobic $C_{6\beta}$ moiety that has been shown to induce conformational change in class A $\beta$-lactamases in the course of turnover.[30]

In contrast to imipenem, a number of clinically used broad-spectrum $\beta$-lactams are effective antibacterials by the virtue of fact that they resist acylation of the active-site serine of class A $\beta$-lactamases. Third-generation cephalosporins such as cefotaxime (**10**) and ceftazidime (**11**), and the monobactam, aztreonam (**12**), are but a few examples of such antibacterials.

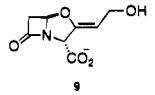


10 11 12

The interactions of these molecules with the active sites of class A $\beta$-lactamases are impaired because of unfavorable steric interactions of the substituents on the $\beta$-lactam rings of these molecules, which retard the formation of the corresponding acyl-enzyme intermediate(s). To put this discussion in perspective, the value for the $K_s$ for ceftazidime (**11**), the aforementioned third-generation cephalosporin, measured for the TEM-1 $\beta$-lactamase is a seemingly unreal value of 10 mM. In contrast, $K_s$ = 3.5 ± 0.1 $\mu$M for imipenem with the wild-type TEM-1 $\beta$-lactamase, indicating that the binding of imipenem to the active site of the enzyme is unencumbered.[31] The $K_s$ values for typical penicillins (*e.g.*, benzylpenicillin or carbenicillin, some of which turn over near the diffusion limit) are <100 $\mu$M, and those for most cephalosporins are in the high-micromolar to low-millimolar range.[9] The low $K_s$ for imipenem argues for the affinity of the enzyme in the formation of the non-covalent enzyme–substrate complex. With a value of 31 $M^{-1}$ $s^{-1}$ for $k_{cat}/K_m$,[32] ceftazidime is a somewhat poorer substrate for the TEM-1 $\beta$-lactamase than is imipenem ($k_{cat}/K_m = 1.5 \times 10^3$ $M^{-1}$ $s^{-1}$), but they are both rightly considered poor substrates for the class A enzymes.[4] However, the mechanistic bases for poor turnover of different molecules such as ceftazidime and imipenem by class A $\beta$-lactamases are entirely distinct.

The observation that unfavorable steric interactions have no bearing on poor turnover of imipenem by class A $\beta$-lactamases is supported by our models for interactions of imipenem at the active site of the TEM-1 $\beta$-lactamase. We note that the effect of slower turnover of imipenem by the TEM-1 $\beta$-lactamase—and perhaps by other class A enzymes such as the *B. cereus* and *S. albus* G enzymes—is manifested largely in $k_{cat}$. Typically, $k_{cat}$ values for the TEM-1 $\beta$-lactamase with good penicillin substrates such as ampicillin or benzylpenicillin range between 1500 and 2000 $s^{-1}$, and for a first-generation cephalosporin (*e.g.*, nitrocefin and cephaloridine) about 500–1500 $s^{-1}$.[9] The $k_{cat}$ value for imipenem with the wild-type enzyme is difficult to estimate because of the biphasic kinetics; however, for the Ser-244 $\beta$-lactamase $k_{cat}$ = 0.04 $s^{-1}$, which is useful as an approximation of the corresponding value for the wild-type

(30) Rizwi *et al.* (Rizwi, I.; Tan, A. K.; Fink, A. L.; Virden, R. *Biochem. J.* **1989**, *258*, 205) investigated the possibility for conformational changes during interactions of clavulanate (**9**)—a $\beta$-lactam molecule without a $C_6$ substituent—with the *S. aureus* $\beta$-lactamase and did not find any.



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(31) For the Ser-244 mutant $\beta$-lactamase $K_s$ = 175 ± 13 $\mu$M. The contribution of Arg-244 to the ground-state binding of the TEM-1 $\beta$-lactamase to imipenem via a hydrogen bond to its side chain—calculated from $\Delta\Delta G = -RT \ln(K_s)_{mut}/(K_s)_{wt}$—is 2.3 kcal/mol.

(32) A detailed analysis of interactions of third-generation cephalosporins with class A $\beta$-lactamases will be published in the future.

enzyme.[33] We see here an *attenuation of $k_{cat}$ as large as 50,000-fold for imipenem.*

Glutamate-166 has been shown to be the general base that activates the structurally conserved water molecule Wat-712 to attack the carbonyl of the acyl-enzyme intermediates for typical substrates of class A β-lactamases.[34] Interestingly, the methyl group of the hydroxyethyl function at $C_{6\alpha}$ of imipenem displaces Wat-712 from the active site on energy minimization (Figure 1). Similarly, it has been shown that in the *precatalytic complex* this water molecule is displaced by imipenem binding to the active site of the *S. albus* G enzyme.[21] There is room for Wat-712 to remain coordinated to the side-chain carboxylate of Glu-166, although its position is no longer optimal—*i.e.*, an in-line orientation of the Glu-166 side-chain carboxylate, Wat 712, and ester carbonyl—for a nucleophilic attack at the ester carbonyl. Indeed, the methyl group of the hydroxyethyl moiety occupies essentially the space that Wat-712 would occupy for the acyl-enzyme intermediate of a favorable substrate for β-lactamase. The unique orientation of the $C_{6\alpha}$ hydroxyethyl group of imipenem allows for the displacement of the hydrolytic water molecule from its optimal position upon acylation of the active-site serine and, as a consequence, results in retardation of the rate of hydrolysis of the two acyl-enzyme intermediates. Intuitively, it may appear reasonable that the hydroxyl of the hydroxyethyl group should displace the water molecule. However, the significant interaction that orients the $C_{6\alpha}$ moiety appears to be the strong hydrogen bond between the side chain of Asn-132 and the hydroxyl of the hydroxylethyl group, which directs the methyl group into the space that Wat-712 would normally occupy.

In conclusion, we have demonstrated that the mechanism of turnover of imipenem is more complicated than previously thought. The existence of both a conformational change during turnover, and reasonably stable enzyme−product complex(es) for imipenem was demonstrated. Whereas we showed that the rate of deacylation is slower than the rate of dissociation of the product(s) from the active site, we still do not have a fully satisfactory structural explanation for the differences between the rates of deacylation for the two acyl-enzyme intermediates (**2** and **3**) beyond their interactions with the β-hydroxyl of Ser-130 (*vide supra*). However, the structural insight associated with interactions of the $C_{6\alpha}$ hydroxyethyl group with the enzyme active site explains the basis for the slow rate of turnover of imipenem. The disclosure of these interactions here may prove helpful in future design of β-lactam antibiotics.[35] Despite the widespread clinical use of imipenem, this β-lactam antibiotic has been relatively free from cases of emergent resistance. However, we anticipate that mutations in the genes for class A β-lactamases which would affect the ability of the enzyme to undergo the conformational change or destabilize the enzyme−product complexes—such as the Ser-244 TEM enzyme described previously by us[7]—would minimize the inhibition of the enzyme by imipenem, or even potentially enhance turnover.

**Acknowledgment.** This work was supported in part by a grant from the National Institutes of Health and a predoctoral fellowship from the Philippe Foundation (to P.T.). We thank Dr. Ichiro Shinkai of Merck and Co. for providing us with a generous sample of imipenem and Professor Michael James for making the crystal coordinates of the TEM-1 β-lactamase available to us. Modeling was carried out by Dr. Young June Cho.

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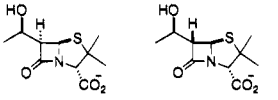
(33) The value of $k_{cat}$ for the more rapid phase of turnover by the wild-type TEM-1 β-lactamase may be as much as 10-fold faster if the contribution of the hydrogen bond by the Arg-244 side chain is taken into account.

(34) Adachi, H.; Ohta, T.; Matsuzawa, H. *J. Biol. Chem.* **1991**, *266*, 3186. Escobar, W. A.; Tan, A. K.; Fink, A. L. *Biochemistry* **1991**, *30*, 10783.

(35) For experimental demonstration of the importance of the interactions of the $C_{6\alpha}$ hydroxyethyl group with the enzyme active site for slow turnover, we synthesized compounds **13** and **14** which possess the penicillanic acid nucleus. The $C_6$ hydroxyethyl group has the configurations β and α in compounds **13** and **14**, respectively. The TEM-1 β-lactamase turned over **13** with $k_{cat}/K_m = 2.8 \times 10^5\ M^{-1}\ s^{-1}$; the corresponding value for turnover of compound **14** was as much as $10^4$-fold slower. These results clearly demonstrate that the slow turnover of imipenem is primarily due to the function of the $C_{6\alpha}$ hydroxyethyl group and not the carbapenem nucleus. The syntheses and kinetic analyses of these compounds, along with a series of other related compounds, will be published later (Miyashita and Mobashery, unpublished results).

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