β-Secondary and Solvent Deuterium Kinetic Isotope Effects on β-Lactamase Catalysis†

S. A. Adediran, S. A. Deraniyagala, Yang Xu, and R. F. Pratt*

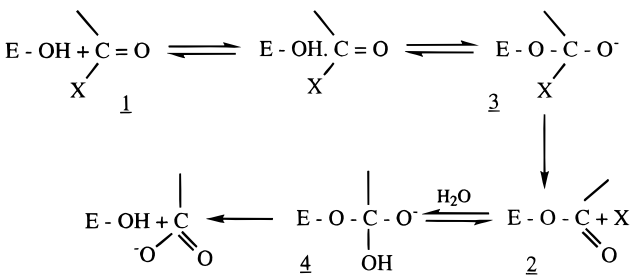
Department of Chemistry, Wesleyan University, Middletown, Connecticut 06459

Received September 5, 1995; Revised Manuscript Received January 2, 1996⊗

ABSTRACT: β-Secondary and solvent deuterium kinetic isotope effects have been determined for the steady-state kinetic parameters *V*/*K* and *V* for turnover of a depsipeptide substrate, *m*-[[(phenylacetyl)glycyl]-oxy]benzoic acid, and of a β-lactam substrate, penicillanic acid, by three typical class A β-lactamases and a class C β-lactamase. The isotope effects on alkaline hydrolysis of these substrates have been used as a frame of reference. The effect of the transition state conformation of the substrates in determining the β-secondary isotope effects has been explicitly considered. The inverse β-secondary isotope effects on both *V*/*K* and *V* for the class A enzymes with both substrates indicate transition states where the carbonyl group of the scissile bond has become tetrahedral and therefore reflect typical acyl-transfer transition states. The solvent isotope effects indicate that enzyme deacylation (as reflected in V for the *Staphylococcus aureus* PC1 β-lactamase) may be a classical general-base-catalyzed hydrolysis but that there is little proton motion in the enzyme acylation transition state (as revealed by *V*/*K*) for the TEM β-lactamase and *Bacillus cereus* β-lactamase I. These results provide kinetic support for the conjecture made on structural grounds that class A β-lactamases employ an asymmetric double-displacement mechanism. The isotope effects on *V*/*K* for the class C β-lactamase of *Enterobacter cloacae* P99 suggest an acyl-transfer transition state for the penicillin, although, as for the class A enzymes, without significant proton motion. On the other hand, the *V*/*K* transition state for depsipeptide does not seem to involve covalent chemistry. Suggestive of this conclusion are the measured β-secondary isotope effect of 1.002 $\pm$ 0.012 and the inverse solvent isotope effect. These results provide an example of a significant difference between the kinetics of turnover of a β-lactam and a depsipeptide by a β-lactamase. The V transition state for both substrates with the P99 β-lactamase probably involves acyl-transfer (deacylation) where the conformation of the acyl-enzyme is closely restricted. The conformations of acyl-enzymes of the PC1 and P99 β-lactamases correlate to the (different) dispositions of general base catalysts at their active sites.

β-Lactamases catalyze the hydrolysis of β-lactam antibiotics and of a variety of acyclic depsipeptides. There has been much recent discussion, fueled by a variety of experimental and theoretical approaches and in particular by the increasing availability of atomic resolution crystal structures, on the mechanism(s) of catalysis employed by these enzymes. The reactions are well-known (Waley, 1992; Govardhan & Pratt, 1987) to involve a double-displacement mechanism which can be minimally represented by Scheme 1. The active-site serine hydroxyl group, acting as a nucleophile, attacks the carbonyl group of the scissile bond of the substrate, **1**, leading to a covalent acyl-enzyme intermediate, **2**, which is then hydrolyzed. Each of the two acyl-transfer steps, enzyme acylation and deacylation, is generally believed to proceed through a tetrahedral intermediate, **3** and **4**, respectively, and to be catalyzed by auxiliary functionality of the active site. One would expect proton transfers in particular to require additional catalysis, but the molecular nature of this process is still unclear. In class A β-lactamases, the conserved active-site residues Glu-166, Lys-73, and Ser-130 have been assigned various roles in proton transfer catalysis, while in class C enzymes the corresponding Lys-67 and Tyr-150 (there is no analog of Glu-166 in class C β-lactamases) have been similarly proposed. See, for example, the discussion provided by Rahil & Pratt (1994), Lobkovsky et al. (1994), and Vijayakumar et al. (1995).

Scheme 1



A related aspect of the problem is the question of the nature of the transition states corresponding to the steady-state parameters *V*/*K* (k_{cat}/K_m) and *V* (k_{cat}) which characterize the catalyzed reaction at low and high substrate concentrations, respectively. Since there is generally no close relationship between these steady-state parameters for turnover of substrates by β-lactamases and the susceptibility of these substrates to nucleophiles (Govardhan & Pratt, 1987; Frère et al., 1988; Xu et al., 1996, the accompanying paper) despite the mechanism of Scheme 1, there are certainly questions as to whether all features of the mechanism are identical for all substrates (Pazhanisamy & Pratt, 1989;

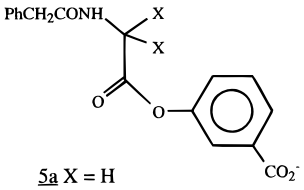
† This research was supported by the National Institutes of Health, Grant AI-17986, and by National Science Foundation Grants INT-9107630 and INT-9211971.

⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1996.

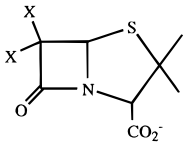
0006-2960/96/0435-3604$12.00/0

Murphy & Pratt, 1991; Xu et al., 1994). These questions apply equally well to the bacterial DD-peptidases, the evolutionary antecedents of the β-lactamases.

Kinetic isotope effects represent a sensitive method of investigation of these issues (Cook, 1991). In order to begin an application of these methods, we have synthesized the substrates **5** and **6**. A comparison of the steady-state rate



5a X = H
5b X = D



6a X = H
6b X = D

parameters of the protonated substrates (a) with those of the respective deuterated substrates (b) permits determination of β-secondary deuterium kinetic isotope effects. In addition, deuterium solvent kinetic isotope effects on these parameters were obtained. These measurements give direct information on the structure of the above-mentioned transition states for representative class A β-lactamases and a class C β-lactamase. The β-secondary isotope effects can be interpreted in terms of the hybridization of the carbonyl carbon of the scissile bond, i.e., the degree of bond formation to an attacking nucleophile in the transition state, and the dihedral angle between the β-C−X bonds and the carbonyl group which dictates the degree of hyperconjugation between them. The solvent isotope effects give a measure of the extent of labile proton motion in the transition states and are thus indicative of the presence of classical general acid/base catalysis. Taken together, these measurements should give a strong indication of whether the kinetically significant transition states involve the covalent steps of acyl-transfer chemistry or whether only physical processes are involved. The results of these experiments will subsequently be extended, for further comparison and contrast, to the DD-peptidases.

EXPERIMENTAL PROCEDURES

Materials. The β-lactamase I of *Bacillus cereus* (strain 569/H/9) and the β-lactamases of the TEM-2 plasmid (from *Escherichia coli*, strain W3310) of *Staphylococcus aureus* PC1 and of *Enterobacter cloacae* P99 were purchased from the Centre for Applied Microbiology and Research (Porton Down, England) and used as supplied. Penicillanic acid (**6a**) and [6-$^2H_2$]penicillanic acid (**6b**) were prepared as described elsewhere (Deraniyagala et al., 1995). Deuterium oxide (99.9 atom % ^{2}H) and sodium deuteroxide (40 wt %) solution in ^2H_2O (99.9 atom % ^{2}H) for the solvent isotope effects were obtained from Aldrich Chemical Co.

*m-[[N-(Phenylacetyl)[$^2H_2$]glycyl]oxy]benzoic Acid (***5b***)*. Perdeuterioglycine was prepared as described by Stein et al. (1983) from the hydrolysis of diethyl acetamidomalonate (5 g) in 100 g of a ^{2}HCl solution in ^2H_2O obtained by the dilution of 20 g of commercial (Aldrich) deuterium chloride (37 wt %, 99.5 atom % ^{2}H) with ^2H_2O (99.9 atom % ^{2}H; Aldrich). The reaction flask had previously been rinsed with ^2H_2O and dried. The product was dried *in vacuo* and converted into *N*-[(benzyloxy)carbonyl][$^2H_2$]glycine by treatment with benzyl chloroformate in aqueous base (Bodanszky & Bodanszky, 1984). The resulting material (2.7 g), also dried *in vacuo*, was equilibrated overnight in 81 mL MeO^{2}H (99.5 atom %; Aldrich) and reisolated as the tetradeuterio species by evaporation of solvent under vacuum. Similarly deuterated by exchange in 45 mL of MeO^{2}H was 2.95 g of benzyl *m*-hydroxybenzoate. The glassware involved in these and subsequent steps was ^2H_2O rinsed and oven-dried. The deuterated *N*-(benzyloxy)glycine (2.7 g) in dry ethyl acetate (45 mL) was then reacted with dicyclohexylcarbodiimide (2.62 g) and the deuterated benzyl *m*-hydroxybenzoate, the latter in a further 10 mL of dry ethyl acetate. After the mixture had been stirred at 40 °C for 30 min and at 25 °C for 90 min, the dicyclohexylurea was removed by filtration. The ethyl acetate layer was washed twice each with sodium bicarbonate solution and water, dried over $MgSO_4$, and evaporated to dryness, yielding 4.8 g of benzyl *m*-[[*N*-[(benzoyloxy)carbonyl][$^2H_2$]glycyl]oxy]benzoate of sufficient purity, as judged by ^{1}H NMR spectroscopy, to proceed without purification.

The benzyl protecting groups were then removed by hydrogenation of the benzyl ester (1.8 g) at room temperature in dry ethyl acetate (100 mL) over 10% Pd/C (1.8 g) and 2 atm of hydrogen gas for 0.75 h. The product, *m*-([$^2H_2$]-glycyloxy)benzoic acid, 0.85 g, precipitated as a zwitterion. This material was extracted into 10 mM HCl, the Pd/C removed by filtration, and the product isolated by lyophilization.

Phenylacetylation of the amino acid (0.35 g), suspended in 11 mL of methylene chloride, was achieved by reaction at room temperature with phenylacetyl chloride (0.34 mL) in the presence of triethylamine (0.63 mL) for 80 min. After workup in aqueous acid, the isolated product was dried and recrystallized from benzene/ethyl acetate (1/1 v/v), yielding 0.16 g. The melting point and ^{1}H NMR spectrum of this material were essentially identical to those previous reported for the dihydro analog (Govardhan & Pratt, 1987) with the exception of the virtual absence of the glycyl methylene resonance at 4.29 ppm. Careful integration of the 300 MHz ^{1}H NMR spectrum indicated a protium content of 0.7% at this position.

The dihydro analog, *m*-[[*N*-(phenylacetyl)glycyl]oxy]-benzoic acid (**5a**), was prepared as described previously (Govardhan & Pratt, 1987).

Kinetic Methods. *(A) Enzyme-Catalyzed Reactions*. The rates of enzyme-catalyzed hydrolysis of **5** and **6** were measured spectrophotometrically by means of either a Hewlett Packard HP8452 or a Perkin Elmer Lambda 4B spectrophotometer. The wavelengths thus employed were 290 nm ($\Delta\epsilon$ = 1760 M^{-1} cm^{-1}) and 300 nm ($\Delta\epsilon$ = 590 M^{-1} cm^{-1}) for **5** and between 230 and 240 nm ($\Delta\epsilon$ = 700 M^{-1} cm^{-1} at 230 nm) for **6**. All reactions were carried out at 25.0 °C, where the temperature was maintained at a constant value by either a Peltier junction accessory (HP89090A) or a Lambda RM6 thermostated water bath; thermistor probe measurements indicated that the temperature remained constant during and between kinetic runs to within 0.1 °C. Steady-state parameters for the enzyme-catalyzed reactions were obtained from measurements of initial rates as a function of substrate concentration by the method of Wilkinson (1961). All enzyme-catalyzed reactions were studied in 20 mM MOPS buffer at pH or pD 7.5, unless otherwise noted. This essentially represents the pH of maximal activity of the enzymes studied except for the PC1 β-lactamase where the maximum is at lower pH (Anderson

et al., 1983). Data at pH = pD = 6.0 (20 mM MES buffer) are also reported for this enzyme. Values of pD were determined by addition of 0.40 to glass electrode measurements (Glasoe & Long, 1960). Enzyme concentrations in stock solutions were determined spectrophotometrically (Murphy & Pratt, 1991). A value of 7.1×10^4 M^{-1} cm^{-1} (Joris et al., 1985) was taken for the extinction coefficient of the *E. cloacae* P99 β-lactamase at 280 nm. In cases where dilution of the β-lactamases below 0.1 μM occurred, 0.1% or 0.5% bovine serum albumin was included in buffers for protein stabilization (Persaud et al., 1986; Christensen et al., 1990).

In order to test for slow product dissociation from the P99 β-lactamase, mixtures of enzyme (8.0 μM in final solution) and product, (phenylacetyl)glycine from **5** or penicilloate from **6** (the latter prepared *in situ* by enzymatic hydrolysis of **6** immediately prior to the kinetics experiment), at enzyme-saturating concentrations (after mixing) of 100 and 8.5 mM, respectively (K_i values were estimated from measured rates to be >10 mM and 0.14 mM respectively), were mixed with equal volumes of a solution of the substrate cephalothin (final concentration 2.0 mM) employing a Durrum D110 stopped-flow spectrophotometer. The absorbance of the ensuing reaction mixtures was followed at 320 nm (cephalosporoate) at times commencing at about 5 ms after mixing.

(B) Isotope Effects on V/K. Values of *V/K* for **5** and **6** and the various enzymes were obtained from the pseudo-first-order rate constants of enzyme-catalyzed reactions at low concentrations ($\leq 0.1K_m$) of the respective substrates. In typical experiments, for both the secondary and solvent isotope experiments, 0.1 mL aliquots of concentrated stock solutions of the enzyme in buffer were added by thermostated syringe to 3.35 mL aliquots of stock solutions of the substrate in a thermostated cuvette in the spectrophotometer. Subsequent reactions were followed for at least six half-lives and the computer-acquired readings of absorbance *vs* time used to calculate pseudo-first-order rate constants by means of a nonlinear squares program which treated the final absorbance and the rate constant as adjustable parameters. Generally, kinetic runs alternated between the two isotopes and kinetic isotope effects were calculated from successive pairs and averaged. For secondary isotope effects, the same enzyme stock solution was used, and separate substrate solutions were prepared. For the solvent isotope effects, a common enzyme solution in 1/1 (v/v) $H_2O/^2H_2O$ buffer, glass electrode reading 7.3, was employed and, again, separate substrate solutions of the protiosubstrate in H_2O or ^2H_2O. A small linear correction was applied to the rate constants involved in measurement of the solvent isotope effects to extrapolate the values to 100% H_2O and 100% ^2H_2O.

In two cases, those of **6** and the P99 and TEM β-lactamases, it was not possible to study the hydrolysis reaction at concentrations of **6** less than $0.1K_m$ because of the small absorption changes under these conditions. This problem was overcome by the addition of sufficient amounts of a competitive inhibitor to increase the apparent K_m 10-fold, and thus correspondingly higher substrate concentrations could be used. For the P99 β-lactamase, [(3-dansylamido)-phenyl]boronic acid, $K_i = 0.6$ μM (Dryjanski & Pratt, 1995), was employed, and for the TEM enzyme boric acid itself was used; the K_i in the latter case was determined to be 1.8 mM from measurements of boric acid inhibition of the hydrolysis of **5a**. These inhibitors, when used, were included in either the substrate or enzyme stock solution so that an additional volumetric measurement was not needed. Correction was made to the apparent solvent kinetic isotope effect for the solvent isotope effects on K_i. According to eq 1 (where $^D(V/K)_{app}$ is the apparent isotope effect in the

$$^D(V/K) = {}^D(V/K)_{app}/{}^DK_i \quad (1)$$

presence of the inhibitor and ^DK_i is the solvent isotope effect on the K_i of the inhibitor), these were found to be 1.54 ± 0.08 for inhibition of the P99 enzyme by [(3-dansylamino)-phenyl]boronate and 1.18 ± 0.07 for inhibition of the TEM β-lactamase by borate.

(C) Isotope Effects on V. These were determined in much the same way as described above. In general, 0.10 mL portions of enzyme in the appropriate buffer were added to 0.90 mL aliquots of the substrates. For solvent isotope effects, the enzyme solutions contained 1/1 (v/v) $H_2O/^2H_2O$ and the substrate solutions H_2O or ^2H_2O. Initial rates were determined by linear least squares fitting of absorbance readings at low substrate conversion. In cases where, for practical reasons, the rates could not be measured under completely saturating conditions, the method of Stein (1983), as expressed in eq 2, was employed to calculate the ^DV for

$$^DV = {}^D\upsilon(1 + K_m/S_0^H) - {}^DV/K(K_m/S_0^D) \quad (2)$$

the isotope effect on measured rates, $^D\upsilon$, and $^D(V/K)$; in eq 2, S_0^H and S_0^D are the initial protonated and deuterated substrate concentrations, respectively, and K_m is the Michaelis constant for substrate under the reference (hydrogen) conditions.

(D) Isotope Effects in Alkaline Hydrolysis. The alkaline hydrolysis of **5** was also studied spectrophotometrically as described above. Pseudo-first-order rate constants of hydrolysis were obtained in solutions containing 4 mM lyoxide with an ionic strength of 1.0 (KCl).

Molecular Modeling. A model of **5** was constructed by means of the Builder module of Insight II, version 2.2.0 (Biosym Technologies, San Diego, CA), run on an IBM RISC/6000 Model 530H computer. Conformational energy calculations and minimization were performed by the Discover Molecular Simulation Program, version 2.9.

RESULTS AND DISCUSSION

The depsipeptide **5** and the β-lactam **6** are substrates of serine β-lactamases which catalyze their hydrolysis (Govardhan & Pratt, 1987; Deraniyagala et al., 1995). Steady-state parameters for their turnover by three class A β-lactamases and a typical class C β-lactamase are given in Table 1. Comparison of the values of these parameters with those of other β-lactamase substrates (Govardhan & Pratt, 1987; Galleni & Frère, 1988; Galleni et al., 1988; Matagne et al., 1990) suggests that **5** and **6** are, in general, modest substrates of these enzymes. They are substantially poorer than the best-known substrates, which essentially achieve the level of perfection (Hardy & Kirsch, 1984; Christensen et al., 1990), but rather better than a wide range of other substrates which act as mechanism-based inhibitors of β-lactamases (Pratt, 1992). One would anticipate that chemical steps would be rate-determining to their turnover by the enzyme.

Alkaline Hydrolysis. The alkaline hydrolysis of **5** and **6** was studied as a model reaction involving nucleophilic

Table 1: Steady-State Parameters for Hydrolysis of **5** and **6** by β-Lactamases[a]

	5			**6**		
enzyme[b]	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
TEM	25.4 ± 1.7	2.2 ± 0.3	1.2×10^4	23 ± 3	0.21 ± 0.02	1.1×10^5
BC1	1.6 ± 0.3	21.2 ± 4.8	75	6.0 ± 0.4	1.38 ± 0.16	4.4×10^3
PC1	0.042 ± 0.006	≤ 0.1	≥ 420	*c*	*c*	*c*
P99	125 ± 4	0.23 ± 0.02	5.43×10^5	2.9 ± 0.5	0.24 ± 0.04	1.2×10^4

[a] 20 mM MOPS buffer, pH 7.5, 25 °C. [b] TEM, the β-lactamase of the TEM plasmid of *E. coli*; BC1, *B. cereus* β-lactamase I; PC1, the β-lactamase of the PC1 plasmid of *S. aureus*; P99, the β-lactamase of *E. cloacae* P99. [c] Not determined.

cleavage. The mechanism and transition states of this reaction are relatively well-understood so that the corresponding kinetic isotope effects could serve as a frame of reference for the results from the enzyme-catalyzed reactions. Second-order rate constants for the hydroxide ion-catalyzed hydrolysis of **5** and **6** were 26 s^{-1} M^{-1} and 3.9×10^{-3} s^{-1} M^{-1}, respectively. A comparison of these values with those of Table 1 serves to emphasize the point made in the introduction on the absence of a general relationship between chemical reactivity and β-lactamase catalysis.

The substrates **5** and **6** appeared well-behaved when their alkaline hydrolysis transition states were probed by β-secondary and solvent deuterium substitutions. β-Secondary kinetic isotope effects (k_H/k_D) for **5** and **6** were 0.942 ± 0.021 (average of six determinations) and 0.948 ± 0.012 (Deraniyagala et al., 1995), respectively. Such values are typical of those found in ester (Hogg, 1978) and amide (Deraniyagala et al., 1995) hydrolysis and indicate the significantly tetrahedral transition state [the β-CH bonds tighten through loss of hyperconjugation to the carbonyl group (Melander & Saunders, 1980)] in a typical $B_{AC}2$ mechanism with a tetrahedral intermediate.

Interpretation of the above numbers in terms of transition state structure is not entirely straightforward since both the angular dependence of hyperconjugation and the inductive effect of the deuterium must be considered (Sunko et al., 1977; Melander & Saunders, 1980) and a reference value where hyperconjugation is completely lost is needed. Since β-secondary isotope effects in the hydrolysis of acyl derivatives arise through hyperconjugation of the electrons of the α-CH bond with the π orbital of the carbonyl group, the effect will depend on the overlap of this C−H bond with the π orbital, i.e., on the dihedral angle θ between the C−H bond and the carbonyl carbon p orbital.

The reference value can be obtained most directly from data on the equilibrium addition of methanol to [$^2H_6$]acetone where a secondary isotope effect, K_H/K_D, of 0.78 has been measured (Jones & Bender, 1960); the same value was also obtained from equilibrium addition of bisulfite to acetone (Geneste et al., 1971). An equation to calculate the β-secondary isotope effect $(K_H/K_D)_0$ for one hydrogen of a freely rotating methyl group if it were fixed at the optimum position, i.e., $\theta = 0°$, was presented by Sunko et al. (1977):

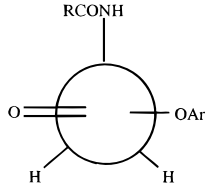
$$\ln(K_H/K_D)_{CH_3} = {}^3/_2 \ln(K_H/K_D)_0 + 3 \ln(K_H/K_D)_i \quad (3)$$

In this equation, $(K_H/K_D)_{CH_3}$ represents the experimentally measured trideuteriomethyl group effect and $(K_H/K_D)_i$ the inductive effect of a single deuterium substituent; the latter is assumed to have no angular dependence. The inductive effect can be estimated from application of the Taft equation (eq 4), where σ^* and ρ^*, respectively, are the usual Taft

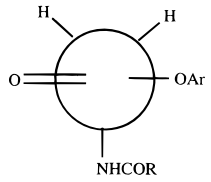
$$\ln(K_H/K_D)_i = -2.303\, \rho^* \sigma^* \quad (4)$$

substituent constant, reflecting in this case the inductive effect of an α-deuterium substituent, and the reaction constant, here for equilibrium nucleophilic addition to substituted acetones. A value of 0.0129 is obtained for $\ln(K_H/K_D)_i$ when values of −0.0033 and 1.7 are substituted for σ^* (Deraniyagala et al., 1995) and ρ^* [from the hydration of ketones (Greenzaid et al., 1967)], respectively. Equation 3 then yields a value of 0.89 for $(K_H/K_D)_0$.

For direct comparison with the above reference value of $(K_H/K_D)_0$, a value of $(k_H/k_D)_0$ for the alkaline hydrolysis of **5** must be calculated, also taking into account the angular dependence of hyperconjugation and the inductive effect of deuterium. This is less straightforward than the calculation above because, even given that rotation is possible about the carbonyl carbon−Cα bond, the rotamers will be of different energy, i.e., will contribute differently to the observed isotope effect; presumably (and confirmed by molecular mechanics calculations) the stablest rotamers would approximate **7** and **8** (which are not optimal for



7



8

hyperconjugation). Equations 4, 5 (Sunko et al., 1977) and 6, were therefore used for calculations. The angle 114°

$$\ln(k_H/k_D)^{\theta}_{CH_2} = [\cos^2\theta + \cos^2(\theta + 114°)] \ln(k_H/k_D)_0 + 2 \ln(k_H/k_D)_i \quad (5)$$

$$\ln(k_H/k_D)_{CH_2} = \sum_0^{360} \left(\frac{\ln(k_H/k_D)^{\theta}_{CH_2} e^{-\Delta G_\theta/RT}}{\sum_0^{360} e^{-\Delta G_0/RT}} \right) \quad (6)$$

represents the difference in dihedral angle θ between the two α-CH bonds in the energy-minimized structure of **5**. The inductive contribution [$\ln(k_H/k_D)_i = 0.0157$] was calculated from eq 4, $\sigma^* = -0.0033$ and $\rho^* = 2.06$ [from the alkaline hydrolysis of phenyl acetates (Bruice et al., 1970)]. The isotope effect at any dihedral angle θ could then be calculated from eq 5 and given a value for $(k_H/k_D)_0$. The weighted average value of the isotope effect $(k_H/k_D)_{CH_2}$ over all θ could then be calculated from eq 6. In practice, the sum was calculated from $\ln(k_H/k_D)^{\theta}_{CH_2}$ values at 10° intervals. The conformational energies at different θ values were calculated

Table 2: β-Secondary Deuterium Kinetic Isotope Effects in β-Lactamase-Catalyzed Hydrolyses of **5** and **6**

	5		**6**	
enzyme	$^{\beta-D}V/K$	$^{\beta-D}V$	$^{\beta-D}V/K$	$^{\beta-D}V$
TEM	0.974 ± 0.008 (8)[a]	*b*	0.926 ± 0.009 (7)[e]	*b*
BC1	0.949 ± 0.015 (9)	*b*	0.927 ± 0.012 (5)	*b*
PC1	*b*	0.878 ± 0.012 (8) 0.812 ± 0.016 (8)[d]	*b*	*b*
P99	0.998 ± 0.011 (8)[c] 1.003 ± 0.014 (8)[c] 1.004 ± 0.008 (8)[c] av 1.002 ± 0.012 (24)	1.018 ± 0.025 (10)[c] 0.979 ± 0.029 (10)[c] 0.986 ± 0.025 (10)[c] av 0.994 ± 0.026 (30)	0.88 ± 0.05 (8)[f]	1.017 ± 0.027 (9)[c] 1.019 ± 0.026 (8)[c] av 1.018 ± 0.027 (17)

[a] The values given are means with standard deviations; the number of pairs of kinetic runs is given in parentheses. [b] Not determined. [c] Separate experiments. [d] pH 6.0. [e] Determined in 25 mM boric acid (see text). [f] Determined in 20 μM [(3-dansylamido)phenyl]boronic acid (see text).

Scheme 2

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\,S \xrightarrow{k_2} E\text{-}S \xrightarrow{k_3} E + P$$

as described in the Experimental Procedures. It was found that an averaged value equal to that observed experimentally (0.942) could be achieved from a $(k_H/k_D)_0$ value of 0.896. Comparison of this value with that of the carbonyl addition equilibrium $(K_H/K_D)_0$ suggests that the transition state of alkaline hydrolysis of **5** must be strongly tetrahedral and closely resemble the tetrahedral intermediate, in accord with the Hammond postulate.

It is noticeable that β-secondary isotope effects for other aryl acetates (Kovach et al., 1980) appear to have less inverse values than does **5**, but these have better leaving groups and/or less electrophilic acyl groups and may hydrolyze by way of a more concerted mechanism and a less tetrahedral transition state (Hengge & Hess, 1994). With poorer leaving groups and/or nucleophiles, more strongly inverse effects, comparable to those obtained here for **5**, have been observed (Bender & Feng, 1960; Kovach et al., 1980).

A similar calculation for the alkaline hydrolysis of **6** yielded a value of $(k_H/k_D)_0$ of 0.96 (Deraniyagala et al., 1995). This might be interpreted in terms of a somewhat more reactant-like transition state for **6** than for **5**, but one cannot be certain of this since the reference equilibrium isotope effect is not available for the cyclobutanone system. It seems quite likely in fact that $(k_H/k_D)_0$ for cyclobutanone would be significantly less inverse than for an acyclic system because of less hyperconjugation in the ground state—incorporation of a second sp^2 hybridized carbon in the four-membered ring would likely be much less favorable than in the acyclic analog.

The solvent isotope effects on the alkaline hydrolysis of these compounds was also well within the range of expectation—0.748 ± 0.018 (average of six determinations) and (0.757 ± 0.009) (Deraniyagala et al., 1995) for **5** and **6**, respectively. These inverse effects reflect the greater nucleophilicity of O^2H^- in ^2H_2O than of OH^- in H_2O and agree with previous results with phenyl acetate (Jencks & Carriuolo, 1960) and β-lactams (Gensmantel et al., 1978; Bowden & Bromley, 1990a,b) where formation of the tetrahedral intermediate is thought to be rate-determining.

The Class A β-Lactamases, TEM, BC1, and PC1. The isotope effects determined in this work are presented in Tables 1 and 2, along with related data from the literature. Given the simple reaction sequence of Scheme 2, where E-S represents the acyl-enzyme intermediate, $V/K = k_1k_2E_0/(k_2 + k_{-1})$. If it is assumed for the moment that $k_{-1} \gg k_2$, $V/K = k_1k_2E_0/k_{-1} = k_2E_0/K_s$. Under these conditions, isotope effects on V/K will represent properties of the transition state for acylation that distinguish it from the free reactants, enzyme, and substrate.

The inverse $^{\beta-D}V/K$ effects for the TEM and BC1 β-lactamases with the depsipeptide substrates **5** suggest loss of α-CH hyperconjugation in the transition state and thus, partly at least, rate-determining covalent chemistry in the step assigned rate constant k_2 in Scheme 2. The isotope effect for the BC1 β-lactamase is essentially the same as that for alkaline hydrolysis, suggesting a close-to-tetrahedral transition state, while that for the TEM enzyme is somewhat less inverse, suggesting something less tetrahedral. There are, however, a number of factors in an enzyme-catalyzed reaction that may complicate any attempt at direct correlation between the secondary isotope effect and the degree of tetrahedrality of the transition state. These include the following: (i) a phase transfer isotope effect (Kovach & Quinn, 1983) which would lead in a hydrophobic active site to an observed *more normal* (or less inverse) isotope effect; (ii) a reduction in overlap between the carbonyl group and the oxygen of the leaving group because of torsional distortion of the ester group on binding would lead to a *more normal* isotopic effect; (iii) a strong electrophilic activation of the ester carbonyl group such as might be provided by the oxyanion hole (Kraut, 1977; Gerlt & Gassman, 1993) would lead to a *more normal* isotope effect; (iv) torsional restriction of the $CO-CH_2$ bond in the active site could lead to *either* a more normal or more inverse isotope effect, depending on the bound conformation; (v) covalent chemistry may not be totally rate-determining to V/K—a "virtual" transition state (Schowen, 1978)—which would lead to a *more normal* isotope effect.

Most of these factors, as noted, would lead to a more normal or less inverse β-secondary isotope effect, as observed with the TEM β-lactamase. Most of them, however, (i)–(iv), would be small/insignificant in a close-to-tetrahedral transition state.

Within experimental uncertainty, the $^{\beta-D}V/K$ values for **6** and the above class A β-lactamases are the same as for alkaline hydrolysis of **6** and thus presumably reflect the covalent chemistry of acylation.

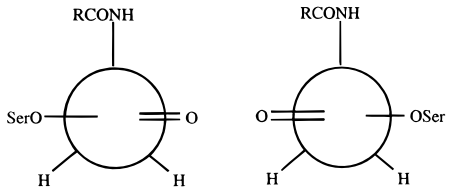
The solvent deuterium isotope effects of the TEM and BC1 enzymes with **5** and **6** are quite striking, particularly when compared with the well-established values for serine proteinases—the latter range from 1.4 to 3.3 for k_{cat}/K_m $(=k_2/K_s)$ for a variety of enzymes and substrates (Elrod et al., 1980; Quinn et al., 1980; Stein et al., 1987) and over a similar range for k_2 (Venkatasubban & Schowen, 1984).

The most direct conclusion from these β-lactamase results in ^2H_2O might be that no protons are in flight ($\varphi^T < 1$) in the transition state; i.e., classical general base catalysis is not involved. Such catalysis normally generates sizably normal (2−4) solvent isotope effects (Johnson, 1966; Alvarez & Schowen, 1987) and is commonly invoked as a major contributor to the effects observed in serine proteinase catalysis (Venkatasubban & Schowen, 1984). The β-lactamase result is thus surprising by analogy with the proteinases and in view of the many suggestions of general-base participation in β-lactamase catalysis (Ellerby et al., 1990; Gibson et al., 1990; Knap & Pratt, 1991), of the elaborate hydrogen-bond network in the β-lactamase active site (Herzberg, 1991; Knox & Moews, 1991) and the extended proton-transfer relays proposed to deliver protons to the leaving group (Lamotte-Brasseur et al., 1991). These solvent isotope effects certainly suggest that protons in the transition state of acylation are spectators to heavy atom rearrangement. The small solvent isotope effects observed, inverse in the case of the TEM enzyme and slightly normal in the case of BC1, could largely reflect medium (Schowen & Schowen, 1982; Kresge et al., 1987; Quinn & Sutton, 1991) or Z (Stein, 1985) isotope effects on substrate binding. The average fractionation factors of the waters of solvation of either the substrate or the active site would then be larger in the enzyme-bound transition state (where some of the water molecules of the active site of the free enzyme would presumably have been released to bulk solvent) than in the ground state of free enzyme and substrate.

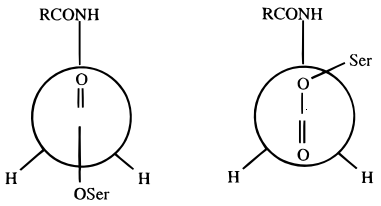
It is possible that the more normal BC1 value does reflect some small degree of active proton participation in the latter enzyme. A somewhat more normal effect is observed in $^DV/K$ for hydrolysis of the penicillin **6** by these enzymes which might, if an inverse medium effect is assumed, be close to the lower end of the range observed in serine proteinase catlaysis. The more normal effect with **6** might reflect the greater need of proton transfer to the (amine) leaving group in this case.

Another possible source of inverse solvent isotope effects has recently been proposed by Cook and co-workers (1995). They suggested that the inverse isotope effects that they observed in the NAD−malic enzyme reaction might arise from the greater viscosity of D_2O than H_2O. In support of this proposal, they showed a comparable inverse effect of viscosity when glycerol and sucrose were used as viscosogens. A viscosity effect, however, does not seem to be responsible for the inverse solvent isotope effect observed on V/K for the reaction between **5** and the TEM β-lactamase. In this instance, $(V/K)_{H_2O}/(V/K)_{glycerol}$ was determined to be (1.12 ± 0.06). The glycerol concentration employed was 9% w/v, which produces a solution of viscosity equal to that of D_2O (Karsten et al., 1995).

Another class A enzyme, the *S. aureus* PC1 β-lactamase, is convenient for the study of isotope effects on V. This arises because of generally low K_m values which in turn derive from the fact that deacylation (k_3) is largely rate-determining under conditions of substrate saturation (Pratt et al., 1988; Govardhan & Pratt, 1987; Virden et al., 1990; Christensen et al., 1990). A dominant factor in determining $^{\beta-D}V$ should be the conformation of the α-methylene group in the acyl-enzyme. The amido side chain of β-lactamase substrates, depsipeptides or β-lactams, is thought to be firmly hydrogen-bonded to the β-strand which forms one wall of the active site and to the side-chain amide of the Asn-132 of the conserved SXN motif (Herzberg & Moult, 1987; Moews et al., 1990; Strynadka et al., 1992; Chen et al., 1993). Since the carbonyl group and the amido side chain would then both be tethered, it seems likely that the α-methylene would be constrained to a small envelope of conformations, and possibly to a single fixed conformation. The expected secondary isotope effect for formation of the tetrahedral intermediate from **5** in deacylation can be calculated from eq 5, assuming $\ln(k_H/k_D)_0 = 0.89$ (a completely tetrahedral transition state) and $\ln(k_H/k_D)_i = 0.0157$ (i.e., that ρ^* for the hydrolysis of the acyl-enzyme is the same as that of the original aryl ester). At one extreme, the conformations **9** and **10** ($\theta \cong 120°$) yield a value of $^{\beta-D}V$ of 0.97, while at



9 10



11 12

the other, **11** and **12** ($\theta \cong 210°$), a value of 0.87 is obtained. The geometry of β-lactamase active sites would suggest that **9** and **11** would be the more likely pair of alternatives. Clearly, the measured isotope effect (0.878 ± 0.012) is much more in accord with an acyl-enzyme conformation resembling **11** (or **12**) than **9** (or **10**). In order to obtain so large an inverse effect, the transition state must also be close to tetrahedral. Somewhat less inverse β-secondary effects observed in the deacylation of the serine proteinases chymotrypsin and elastase were interpreted in terms of earlier transition states (Stein et al., 1983).

The solvent isotope effects on V for the PC1 enzyme for both **5** and benzylpenicillin (Table 3) show clear evidence of proton involvement in the deacylation transition state to an extent generally less than but approaching that seen in serine proteinases (Venkatasubban & Schowen, 1984; Quinn & Sutton, 1991), and which is consistent with a general-base-catalyzed hydrolysis mechanism. The latter is widely believed to obtain in the deacylation of both serine proteinases and β-lactamases.

The smaller but still normal values of the solvent isotope effects on V for the TEM and BC1 enzymes and benzylpenicillin suggest a significant component of deacylation in the V transition state but less so than for PC1. This is in accord with the results of other studies (Christensen et al., 1990). From the limited number of experiments yet performed, it seems that the pH dependence of the solvent isotope effects may be complex. The results reported herein refer only to isotope effects determined at pH values close to those of maximal enzyme activity (see Experimental Procedures). Further experimental investigation of this phenomenon seems warranted.

Table 3: Solvent Deuterium Kinetic Isotope Effects in β-Lactamase-Catalyzed Hydrolyses of **5**, **6**, and Benzylpenicillin

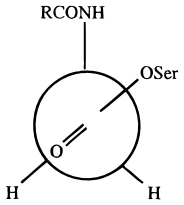
	5		**6**	benzylpenicillin
enzyme	$^{D}V/K$	^{D}V	$^{D}V/K$	^{D}V
TEM	0.70 ± 0.02 (5)[a]		1.04 ± 0.06 (6)[g]	1.39 ± 0.4[h]
	0.72 ± 0.03 (6)[b]	*d*		
	0.61 ± 0.03 (6)[c]			
BC1	1.11 ± 0.02 (3)	*d*	1.23 ± 0.08 (4)	1.6 ± 0.1[h]
				1.67 ± 0.03[i]
PC1	*d*	2.62 ± 0.16 (8)	*d*	1.88 ± 0.15 (10)
		1.66 ± 0.04 (8)[f]		2.42 ± 0.10 (8)[f]
				2.16 ± 0.2[h]
P99	0.79 ± 0.01 (8)[e]	1.44 ± 0.13 (10)	0.99 ± 0.13 (8)[g]	1.59 ± 0.10 (10)
	0.85 ± 0.01 (8)[e]			

[a] The values given are means with standard deviations; the number of pairs of kinetic runs is given in parentheses. [b] pH 7.0. [c] pH 8.0. [d] Not determined. [e] Separate experiments. [f] pH 6.0. [g] Borate correction (see text). [h] Christensen et al. (1990); 0.1 M phosphate, 1 M NaCl, pH 7. [i] Hardy & Kirsh, 1984b; 0.2 M phosphate, 0.02 M pyrophosphate, pH 7−9.

The Class C β-Lactamase of E. cloacae P99. Discussion of the kinetic isotope effects obtained with this enzyme and also presented in Tables 2 and 3 can readily proceed within the framework established above. There are however some interesting differences between these values and those obtained for the class A enzymes. The $^{\beta-D}V/K$ value for the depsipeptide **5**, for example, taking into account the alkaline hydrolysis results, is most simply interpreted to mean that the transition state of the first irreversible step does *not* involve covalent chemistry, i.e., is not the transition state of enzyme acylation. It is possible that a combination of the factors (i)−(iv) described above, perhaps in combination with an early transition state, could also achieve the observed result. The solvent isotope of V/K for **5**, however, does not support the latter possibility, being distinctly inverse and suggesting medium effects rather than general acid/base catalysis. As in the case of the TEM β-lactamase, the deuterium solvent isotope effect seems unlikely to be a viscosity effect. Methanol, to 3 M [relative viscosity 1.3 (Wolf et al., 1981)], had essentially no effect [$(V/K)_{H_2O}/(V/K)_{MeOH} = 1.00 \pm 0.04$] on *V*/*K* (Xu et al., 1996, accompanying paper). Glycerol (9% v/v, relative viscosity 1.24) appeared to have a slightly normal effect [$(V/K_{H_2O})/(V/K_{glycerol}) = 1.20 \pm 0.23$]. Further, the virtual absence of a leaving group effect on *V*/*K* for aryl depsipeptides, including **5** (Xu et al., 1996, accompanying paper), also supports the proposition that acylation chemistry is not present in the *V*/*K* transition rate.

One possible transition state within these limitations would seem to be an irreversible step subsequent to binding but prior to (relatively rapid) acylation, and possibly including a protein conformational change. Diffusion of enzyme and substrate together could not be rate-determining because k_{cat}/K_m is only 5.4×10^5 s^{-1} M^{-1} (Table 1).

An alternative would be the transition state of slow leaving group departure subsequent to reversible acylation. A fast equilibrium protonation of the leaving group prior to departure could contribute to the inverse solvent isotope effect on *V*/*K* for **5**. The latter scenario would require a conformation of the acyl-enzyme that allowed hyperconjugation to a similar extent to that permitted by the free depsipeptide in solution. Such a conformation, **13**, $\theta \cong 90°$, corresponding to one lying between the extremes of **10** and **12**, can be calculated from eq 5. The viscosity data, described above, are probably best seen as inconclusive on the presence of any diffusional component to *V*/*K*.

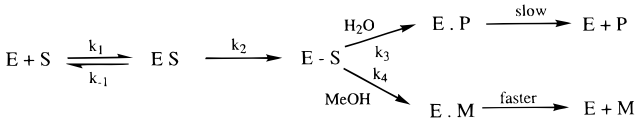


13

In contrast to that above, the *V*/*K* β-secondary isotope effect on the reaction of the P99 β-lactamase with the penicillin **6** gives clear evidence of acyl-transfer chemistry, presumably of the acylation step since the opening of the strained β-lactam ring is very likely to be irreversible. It seems likely therefore that the nature of the transition state of the first irreversible step is different in the turnover by this class C enzyme of a penicillin substrate than in that of a depsipeptide such as **5**. It might be noted in passing that *V*/*K* for the hydrolysis of a good penicillin substrate such as benzylpenicillin by the class A enzymes (Hardy & Kirsch, 1984a; Christensen et al., 1990), and quite likely also by the P99 enzyme in view of k_{cat}/K_m values (Galleni & Frère, 1988), is limited by diffusion together of enzyme and substrate, i.e., $k_{cat}/K_m = k_1$ in Scheme 2; this is not likely to be true for **6** because of the low k_{cat}/K_m values (Table 1). Despite the above indication of a chemical transition state for **6**, the solvent isotope effect is very close to unity and bears much the same relationship to that for **5** as seen in the class A enzymes. The implication here would seem to be of an acyl-transfer transition state with either little proton motion or one where the transition state fractionation factors of protons in motion (presumably <1) are compensated for by reactant fractionation factors.

Finally, the isotope effects on V for the P99 β-lactamase should be considered. The rate-determining step at saturation of the enzyme by these substrates is likely to be deacylation (k_3), for both **5** (Xu et al., 1996, accompanying paper) and **6** (Govardhan & Pratt, 1987; Monnaie et al., 1992). In accord with the latter conclusion, methanol (0−3 M) was observed to accelerate the solvolysis of **6** (1.22 mM) in the presence of the P99 β-lactamase. The solvent isotope effect on V for both **5** and benzylpenicillin (and unlike that on *V*/*K* for **5**) does suggest the presence of an acyl-transfer transition state, but in contrast, the β-secondary isotope effects for both **5** and **6** are very close to unity. The latter values could, in the absence of other evidence, be readily interpreted in terms of a physical rather than chemical transition state. The

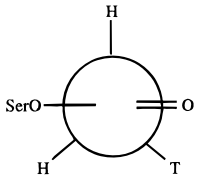
Scheme 3



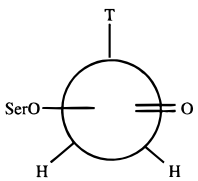
evidence for a rate-determining acyl-transfer process for k_3 is quite strong, however. In particular, the observation of rate acceleration by the alternative nucleophile methanol for **5** (Xu & Pratt, 1996, accompanying paper), **6**, and for benzylpenicillin (Govardhan & Pratt, 1987), and accompanied by formation of appropriate amounts of the respective methanolysis products, has been interpreted in this way. The only obvious alternative, by analogy with the situation of alkaline phosphatase and its alternative phosphoryl acceptor Tris (Hull et al., 1976), might be that of Scheme 3, where the last slow step represents discharge of the product (phenylacetyl)glycine from the enzyme. This slow step could, in principle, be accelerated by the methanolysis, yielding methyl phenaceturate, M, which might be lost more rapidly from the enzyme. This scenario would require a solvent isotope effect of ca. 1.5 on the final step, which could be kinetic in nature, e.g., arising from a protein conformational change accompanying product release, or simply from a (admittedly rather large) medium effect on the released carboxylate (Kresge et al., 1987; Selwood et al., 1993). The (partly) concerted release of a proton from the product might also contribute.

Direct experiment disproves the above scenario, however. No lag phase was observed on mixing solutions containing the P99 β-lactamase and either (phenylacetyl)glycine or penicilloate (the carboxylate or P products from **5** and **6**, respectively) with the reporter substrate cephalothin. In each case, a lag of rate constant k_3 (k_{cat} for the respective substrate) should have been observed if release of P were the slow step.

Alternatively, and perhaps most likely, the transition state for the k_3 step for both **5** and **6** may simply reflect the influence of the conformation of the acyl-enzyme. For depsipeptide **5**, this may resemble **9** where the calculated isotope effect (above) closely approaches the lower (less inverse) limit. A small normal contribution from other factors such as (ii) or (iii) (see above) might be sufficient to complete the picture for this substrate. The situation for **6** is slightly different. The acyl-enzyme conformation analogous to **9** would be **14** (where T represents the 2-thiazolidinyl



14



15

substituent) which would clearly generate a sizable inverse β-secondary isotope effect. A minimal isotope effect, as observed, would be generated by **15**. This could arise by rotation about the C_6-C_7 bond of the penicillin subsequent to acylation. Rotation about the C_5-C_6 also occurs at this time (Kelly et al., 1989; Oefner et al., 1990; Strynadka et al., 1992), and the two motions might be coupled. The conformational motion of the substrate leading to **15** would

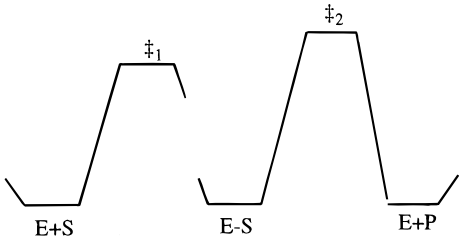


FIGURE 1

not likely occur with a natural penicillin where the binding of its amido side chain would likely keep the acyl-enzyme in a conformation closer to **9**. The conformational change leading to **15** may be responsible to some extent for the slower deacylation of **6** than that of a natural penicillin.

Concluding Discussion. The β-secondary and solvent kinetic isotope effects determined in the experiments described above reveal much about the transition states $\ddagger_1$ (V/K) and $\ddagger_2$ (V in most cases) (Figure 1; E-S represents the acyl-enzyme intermediate and the gap in the line the irreversible acylation step) of steady-state β-lactamase catalysis. Of particular interest are the specific examples of the similarities and differences between the transition states for a depsipeptide and those of a β-lactam, between $\ddagger_1$ and $\ddagger_2$ themselves, between those of a class A and a class C β-lactamase, and between those of serine β-lactamases and serine proteinases.

The most striking difference between the transition states of **5** and **6** was observed with the P99 β-lactamase in $\ddagger_1$ which appears to involve covalent chemistry (a tetrahedral carbon) in **6** but not in **5**. It is possible that the acylation of the enzyme by **5** is so facile—it has a very good leaving group that may not require general acid catalysis—that $\ddagger_1$ involves only noncovalent chemistry, a conformational event, for example. The latter thought is supported by the low leaving group dependence of the aryl phenaceturates (Xu et al., 1996, accompanying paper). In contrast, both transition states of the class A enzyme with both **5** and **6** involve covalent chemistry. The transition state $\ddagger_2$ of the P99 enzyme for both **5** and **6** is probably best interpreted in terms of acyl transfer at present.

An interesting difference between the PC1 (class A) and P99 (class C) enzymes, apart from the nature of $\ddagger_1$, resides in the conformation of the acyl-enzyme. This is probably close to **11** in the PC1 enzyme and to **9** in P99. This difference may well reflect the different positions in space of the functional groups thought to be the general base catalyst of deacylation in the two classes of enzyme, supplied by Glu-166 in class A and Tyr-150 in class C (Herzberg & Moult, 1987; Oefner et al., 1990; Lobkovsky et al., 1994). Inspection of crystal structures of these two enzymes complexed with a phosphonate transition-state analog shows $SerO_\gamma-P-C-NH$ dihedral angles of 35° (65° in a side-chain conformer) and 177° for P99 and PC1, respectively (Lobkovsky et al., 1994; Chen et al., 1993), which certainly correlate well with the proposed conformers **9** and **11**, respectively, for the acyl-enzymes. This finding represents an example of the application of β-secondary deuterium kinetic isotope effects to determine the conformation of the substrate moiety of an enzyme−substrate complex. Sinnott and co-workers (Guo et al., 1994) have recently used β-secondary isotope effects to determine the conformation of transition states in sialidase catalysis—in glycosyl transfer

reactions, hyperconjugation occurs in the transition state rather than the ground state.

The consistent difference between $\ddagger_1$ and $\ddagger_2$ with respect to proton transfer should also be emphasized. Evidence from the solvent isotope effects for some proton motion, suggestive of general base catalysis, was obtained for $\ddagger_2$, but it seems unlikely that there is any in $\ddagger_1$, for *all* enzymes tested, unless their contribution to the observed isotope effects was offset by unusually (Quinn & Sutton, 1991) small fractionation factors in the free enzyme or substrate. A number of active-site water molecules must be displaced on substrate binding (Herzberg, 1991; Knox & Moews, 1991). Loh and Markley (1994) have found a surprising range of fractional factors in the protein amide hydrogens of staphylococcal nuclease, including many less than unity. They also observed changes in specific backbone amide fractionation factors on the binding of an inhibitor to the active site, although the average fractionation factor for the entire protein (60% of the total were surveyed) changed very little. It is possible that a similar variation in fractionation factors may occur within water molecules of the first solvation shell of a protein, particularly at an active site. If the difference in solvent isotope effects between *V*/*K* and *V* is not due to the unusual ground-state fractionation factors, it would then point to the existence of a rather different chemical mechanism for acylation and deacylation of the PC1 β-lactamase. The participation of different functional groups of class A enzymes in these steps—an asymmetric mechanism—has previously been proposed on structural grounds (Herzberg & Moult, 1991).

Finally, it seems that there may be significant differences between the steady-state transition states of serine β-lactamases and proteinases. On the basis of solvent isotope effects, there appears to be rather less proton motion coupled to the heavy atom motion in the β-lactamase. This point should be considered in subsequent proposals regarding β-lactamase mechanisms.

REFERENCES

Alvarez, F. J., & Schowen, R. L. (1987) in *Isotopes in Organic Chemistry* (Bunsel, E., & Lee, C. C., Eds.) Vol. 7, Chapter 1, Elsevier, Amsterdam.

Anderson, E. G., & Pratt, R. F. (1983) *J. Biol. Chem. 258*, 13120−13126.

Bender, M. L., & Feng, M. S. (1960) *J. Am. Chem. Soc. 82*, 6318−6321.

Bodanszky, M., & Bodanszky, A. (1984) *The Practice of Peptide Synthesis*, p 12, Springer-Verlag, New York.

Bowden, K., & Bromley, K. (1990a) *J. Chem. Soc., Perkin Trans 2*, 2103−2109.

Bowden, K., & Bromley, K. (1990b) *J. Chem. Soc., Perkin Trans. 2*, 2111−2116.

Bruice, T. C., Hegarty, A. F., Felton, S. M., Donzel, A., & Kundu, N. G. (1970) *J. Am. Chem. Soc. 92*, 1370−1378.

Chen, C. C. H., Rahil, J., Pratt, R. F., & Herzberg, O. (1993) *J. Mol. Biol. 234*, 165−178.

Christensen, H., Martin, M. T., & Waley, S. G. (1990) *Biochem. J. 266*, 853−861.

Cook, P. F., Ed. (1994) *Enzyme Mechanisms from Isotope Effects*, CRC Press, Boca Raton, FL.

Deraniyagala, S. A., Adediran, S. A., & Pratt, R. F. (1995) *J. Org. Chem. 60*, 1619−1625.

Dryjanski, M., & Pratt, R. F. (1995) *Biochemistry 34*, 3561−3568.

Ellerby, L. M., Escobar, W. A., Fink, A. L., Mitchinson, C., & Wells, J. A. (1990) *Biochemistry 29*, 5797−5806.

Elrod, J. P., Hogg, J. L., Quinn, D. M., Venkatasubban, K. S., & Schowen, R. L. (1980) *J. Am. Chem. Soc. 102*, 3917−3922.

Frère, J.-M., Joris, B., Varetto, L., & Crine, J. (1988) *Biochem. Pharmacol. 37*, 125−132.

Galleni, M., & Frère, J.-M. (1988) *Biochem. J. 255*, 119−122.

Galleni, M., Amicosante, G., & Frère, J.-M. (1988) *Biochem. J. 255*, 123−129.

Geneste, P., Lamaty, G., & Roque, J. P. (1971) *Tetrahedron 27*, 5539−5559.

Gensmantel, N. P., Gowling, E. W., & Page, M. I. (1978) *J. Chem. Soc., Perkin Trans. 2*, 335−342.

Gerlt, J. A., & Gassman, P. G. (1993) *Biochemistry 32*, 11943−11952.

Gibson, R. M., Christensen, H., & Waley, S. G. (1990) *Biochem. J. 272*, 613−619.

Glasoe, P. F., & Long, F. A. (1960) *J. Phys. Chem. 64*, 188−193.

Govardhan, C. P., & Pratt, R. F. (1987) *Biochemistry 26*, 3385−3395.

Greenzaid, P., Luz, Z., & Samuel, D. (1967) *J. Am. Chem. Soc. 89*, 749−756.

Guo, X., Laver, W. G., Vimr, E., & Sinnott, M. L. (1994) *J. Am. Chem. Soc. 116*, 5572−5578.

Hardy, L. W., & Kirsch, J. F. (1984a) *Biochemistry 23*, 1275−1282.

Hardy, L. W., & Kirsch, J. F. (1984b) *Biochemistry 23*, 1282−1287.

Hengge, A. C., & Hess, R. A. (1994) *J. Am. Chem. Soc. 116*, 11256−11263.

Herzberg, O. (1991) *J. Mol. Biol. 217*, 701−719.

Herzberg, O., & Moult, J. (1987) *Science 236*, 694−701.

Herzberg, O., & Moult, J. (1991) *Curr. Opin. Struct. Biol. 1*, 946−953.

Hogg, J. L. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) Chapter 5, Plenum Press, New York.

Hull, W. E., Halford, S. E., Gutfreund, H., & Sykes, B. D. (1976) *Biochemistry 15*, 1547−1561.

Jencks, W. P., & Carriuolo, J. (1960) *J. Am. Chem. Soc. 82*, 675−681.

Johnson, S. L. (1967) *Adv. Phys. Org. Chem. 5*, 237−330.

Jones, J. M., & Bender, M. L. (1960) *J. Am. Chem. Soc. 82*, 6322−6326.

Joris, B., DeMeester, F., Galleni, M., Reckinger, G., Coyette, J., Frère, J.-M., & Van Beeumen, J. (1985) *Biochem. J. 228*, 241−248.

Karsten, W. E., Lai, C.-J., & Cook, P. F. (1995) *J. Am. Chem. Soc. 117*, 5914−5918.

Kelly, J. A., Knox, J. R., Zhao, H., Frère, J.-M., & Ghuysen, J.-M. (1989) *J. Mol. Biol. 209*, 281−295.

Knap, A., & Pratt, R. F. (1991) *Biochem. J. 273*, 85−91.

Knox, J. R., & Moews, P. C. (1991) *J. Mol. Biol. 220*, 435−455.

Kovach, J. M., & Quinn, D. M. (1983) *J. Am. Chem. Soc. 105*, 1947−1950.

Kovach, J. M., Elrod, J. P., & Schowen, R. L. (1980) *J. Am. Chem. Soc. 102*, 7530−7534.

Kraut, J. (1977) *Annu. Rev. Biochem. 46*, 331−358.

Kresge, A. J., More O'Ferrall, R. A., & Powers, M. F. (1987) in *Isotopes in Organic Chemistry* (Bunsell, E., & Lee, C. C., Eds.) Vol. 7, Chapter 4, Elsevier, Amsterdam.

Lamotte-Brasseur, J., Dive, G., Dideberg, O., Charlier, P., Frère, J.-M., & Ghuysen, J.-M. (1991) *Biochem. J. 279*, 213−221.

Lobkovsky, E., Billings, E. M., Moews, P. C., Rahil, J., Pratt, R. F., & Knox, J. R. (1994) *Biochemistry 33*, 6762−6772.

Loh, S. N., & Markley, J. L. (1994) *Biochemistry 33*, 1029−1036.

Matagne, A., Misselyn-Bauduin, A.-M., Joris, B., Erpicum, T., Granier, B., & Frère, J.-M., (1990) *Biochem. J. 265*, 131−146.

Melander, L., & Saunders, W. H., Jr. (1980) in *Reaction Rates of Isotopic Molecules*, Chapter 6, Wiley-Interscience, New York.

Moews, P. C., Knox, J. R., Dideberg, O., Charlier, P., & Frère, J.-M. (1990) *Proteins: Struct., Funct., Genet. 7*, 156−171.

Monnaie, D., Virden, R., & Frère, J.-M. (1992) *FEBS Lett. 306*, 108−112.

Murphy, B. M., & Pratt, R. F. (1991) *Biochemistry 30*, 3640−3649.
Oefner, C., D'Arcy, A., Daly, J. J., Gubernator, K., Charnas, R. L., Heinze, I., Hubschwerlen, C., & Winkler, F. K. (1990) *Nature 343*, 284−288.
Pazhanisamy, S., & Pratt, R. F. (1989) *Biochemistry 28*, 6875−6882.
Persaud, K. C., Pain, R. H., & Virden, R. (1986) *Biochem. J. 237*, 723−730.
Pratt, R. F. (1992) in *The Chemistry of β-Lactams* (Page, M. I., Ed.) Chapter 7, Chapman & Hall, London.
Pratt, R. F., McConnell, T. S., & Murphy, S. J. (1988) *Biochem. J. 254*, 919−922.
Quinn, D. M., & Sutton, L. D. (1991) in *Enzyme Mechanism from Isotope Effects* (Cook, P. F., Ed.) Chapter 3, CRC Press, Boca Raton, FL.
Quinn, D. M., Elrod, J. P., Ardis, R., Friesen, P., & Schowen, R. L. (1980) *J. Am. Chem. Soc. 102*, 5365−5369.
Rahil, J., & Pratt, R. F. (1994) *Biochemistry 33*, 116−125.
Schowen, K. B., & Schowen, R. L. (1982) *Methods Enzymol. 87*, 551−606.
Schowen, R. L. (1978) in *Transition States of Biochemical Processes* (Gandour, R. D., & Schowen, R. L., Eds.) Chapter 2, Plenum, New York.
Selwood, T., Feaster, S. R., States, M. J., Pryor, A. N., & Quinn, D. M. (1993) *J. Am. Chem. Soc. 115*, 10477−10482.
Stein, R. L. (1983) *J. Am. Chem. Soc. 105*, 5111−5116.
Stein, R. L. (1985) *J. Am. Chem. Soc. 107*, 6039−6042.
Stein, R. L., Elrod, J. P., & Schowen, R. L. (1983) *J. Am. Chem. Soc. 105*, 2446−2452.
Stein, R. L., Strimpler, A. M., Hori, H., & Powers, J. C. (1987) *Biochemistry 26*, 1305−1314.
Strynadka, N. C. J., Adachi, H., Jensen, S. E., Johns, K., Sielecki, A., Betzel, C., Sutoh, K., & James, M. N. G. (1992) *Nature 359*, 700−705.
Sunko, D. E., Szele, I., & Hehre, W. J. (1977) *J. Am. Chem. Soc. 99*, 5000−5005.
Venkatasubban, R. S., & Schowen, R. L. (1984) *Crit. Rev. Biochem. 17*, 1−44.
Vijayakumar, S., Ravishanker, G., Pratt, R. F., & Beveridge, D. L. (1995) *J. Am. Chem. Soc. 117*, 1722−1730.
Virden, R., Tan, A. K., & Fink, A. L. (1990) *Biochemistry 29*, 145−153.
Waley, S. G. (1992) in *The Chemistry of β-Lactams* (Page, M. I., Ed.) Chapter 6, Chapman & Hall, London.
Wilkinson, G. M. (1961) *Biochem. J. 80*, 324−332.
Wolf, A. V., Brown, M. G., & Prentiss, P. G. (1981) in *Handbook of Chemistry and Physics* (Weast, R. C., Ed.) 60th ed., pp D227−D276, CRC Press, Boca Raton, FL.
Xu, Y., Soto, G., Adachi, H., van der Linden, M. P. G., Keck, W., & Pratt, R. F. (1994) *Biochem. J. 302*, 851−856.
Xu, Y., Soto, G., Hirsch, K. R., & Pratt, R. F. (1996) *Biochemistry 35*, 3595−3603.

BI952107I