β-Secondary and Solvent Deuterium Kinetic Isotope Effects on Catalysis by the *Streptomyces* R61 DD-Peptidase: Comparisons with a Structurally Similar Class C β-Lactamase†

S. A. Adediran‡ and R. F. Pratt*

Department of Chemistry, Wesleyan University, Middletown, Connecticut 06459

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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 series of acyclic substrates by the DD-peptidase of *Streptomyces* R61 and the class C β-lactamase of *Enterobacter cloacae* P99. Although these enzymes are evolutionarily related and have very similar tertiary and active site structure, they are functionally very different—the former efficiently catalyzes the hydrolysis of β-lactams but not acyclic peptides while vice versa applies to the latter. The measured kinetic isotope effects reveal both similarities and differences in the steady-state transition states for turnover of the various substrates by these enzymes. In most cases, inverse β-secondary isotope effects were observed, reflecting typical acyl-transfer transition states. With one substrate, however, *m*-[[(phenylacetyl)glycyl]oxy]benzoic acid, isotope effects on *V/K* of very close to unity were obtained for both enzymes. These were interpreted in terms of acylation transition state conformations where the extent of β-CH hyperconjugation was similar to that in the free substrate. Differences in deacylation transition states (*V*) between the two enzymes with this substrate were interpreted in terms of different acyl-enzyme conformations. Solvent deuterium kinetic isotope effects on *V/K* were uniformly small, some even inverse, for both enzymes and with all substrates tested. At face value, this suggests the counterintuitive conclusion that little proton transfer occurs in acylation transition states in all of these instances. Closer analysis, however, suggests that for ester and amide (and probably β-lactam) substrates, this result probably arises from an increase in proton fractionation factors on substrate binding being offset by their decrease in the acylation transition state. The former event derives from proton rearrangement on substrate binding and the latter, presumably, from general acid/base catalysis. This result may be general to all β-lactam-recognizing enzymes. The solvent isotope effects also suggest that, at least for the P99 β-lactamase, the acylation transition state of a thioester substrate does not involve proton transfer. This can be interpreted in terms of the rate-determining breakdown of a tetrahedral intermediate where no protonation of the leaving thiolate is required. Deacylation transition states of both enzymes appear to involve significant proton transfer, presumably arising from general acid/base catalysis.

A central issue in the study of β-lactam-recognizing enzymes is that of evolution—both the present-day evolution of β-lactamases and DD-peptidases leading to bacterial resistance toward β-lactam antibiotics (*1*, *2*), and the primeval evolution of β-lactamases from DD-peptidases (*3*, *4*).

An important pair of closely related enzymes that epitomizes the difference between a β-lactamase and a DD-peptidase is that of a class C β-lactamase (e.g., that of *Enterobacter cloacae* P99) and the *Streptomyces* R61 DD-peptidase. These enzymes have very similar tertiary structure and active site composition (*5*−*7*). They have in common the ability to catalyze the hydrolysis of acyclic depsipeptides (*8*−*10*), but differ in that (i) the β-lactamase but not the DD-peptidase effectively catalyzes β-lactam hydrolysis and (ii) the DD-peptidase but not the β-lactamase effectively catalyzes acyclic peptide hydrolysis (*11*).

To compare and contrast and thus to better understand the details of the catalytic features of these active sites, we have measured the β-secondary and solvent deuterium kinetic isotope effects for steady-state turnover of a variety of acyclic substrates, **1**−**7**, of the above two enzymes. The former isotope effect is sensitive to bond-making or -breaking at the scissile acyl group in the transition state, and the latter is sensitive to proton transfer in progress in the transition state such as would be expected in the case of general acid or base catalysis. We have previously used these methods in a comparative study of class A and C β-lactamases employing a β-lactam substrate as well as the acyclic depsipeptide **1** (*12*).

In the present work, to better compare the two enzymes, the motif of the acyclic substrate has been extended beyond the original compound **1,** to the thio esters **2**−**4** and the peptides **5**−**7**. The thio ester was of interest because such compounds appear to be good substrates of the R61 DD-peptidase (*10*) and because previous experiments showed that acylation of the P99 β-lactamase by **2a** was rate-determining at saturation (*13*), thus enabling a direct view of the acylation transition state: under saturating substrate conditions, deacylation of the enzyme is rate-determining in the case of **1** (*13*).

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‡ On leave from the University of Ilorin, Ilorin, Nigeria.

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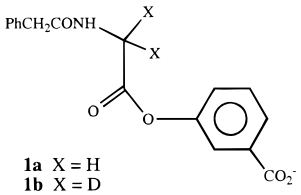

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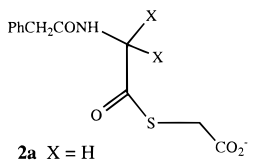

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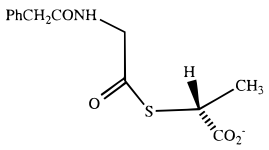

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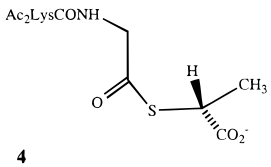

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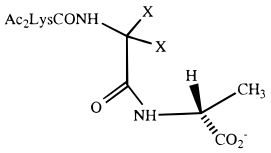

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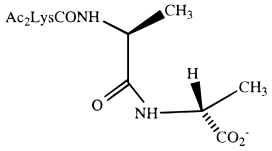

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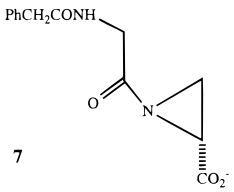

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The thioester **4** was included to assess the effect of changing the side chain from one agreeable to both enzymes (*14*) to one greatly preferred for amide hydrolysis by the R61 DD-peptidase (*11, 15*). Finally, the peptides **5**−**7** were included to determine whether differences were evident between the transition states for amide hydrolysis and those of ester hydrolysis. Peptides are not generally hydrolyzed by the P99 β-lactamase at any appreciable rate, but the *N*-acylaziridine **7**, because of its chemical reactivity and shape—the presence of a tetrahedral β-lactam-like nitrogen in the scissile bond—is a substrate of both enzymes (*16*).

EXPERIMENTAL PROCEDURES

Materials. The β-lactamase of *Enterobacter cloacae* P99 was purchased from the Centre for Applied Microbiology and Research, Porton Down, U.K., and used as received. The DD-peptidase from *Streptomyces* R61 was generously provided by Dr. J.-M. Frère of the University of Liége, Liége, Belgium. The concentrations of stock solutions of the β-lactamase and DD-peptidase were obtained by employment of published extinction coefficients, 7.1×10^4 M^{-1} cm^{-1} (*17*) and 3.9×10^4 M^{-1} cm^{-1} (*18*), respectively. *m*-[[*N*-(Phenylacetyl)glycyl]oxy]benzoic acid **1a** and its specifically dideuterated analogue *m*-[[*N*-(phenylacetyl)-[$^2H_2$]glycyl]oxy]-benzoic acid **1b** were available from the previous study mentioned in the introduction (*12*). Deuterium oxide (99.9 atom % ^2H) and sodium deuteroxide (40 wt %) solution in ^2H_2O (99.9 atom % ^2H) were purchased from Aldrich Chemical Co. D-Phenylalanine and D-leucine were obtained from Sigma.

*(Phenylacetyl)-[$^2H_2$]glycylthioglycolic Acid, **2b**.* Perdeuterioglycine hydrochloride was prepared by the method of Stein et al. (*19*) as previously described (*12*), and converted into *N*-Boc-[^2H]glycine by treatment with di-*t*-butyl pyrocarbonate. Thus, perdeuterioglycine hydrochloride (5.96 g, 50 mmol) was dissolved in water (40 mL), and the pH of the solution was adjusted to 7.0 with 5 M NaOH. Dioxane (100 mL) and 1 M NaOH (50 mL) were then added, and the solution was ice-cooled with stirring. After addition of the pyrocarbonate (12 g, 55 mmol), the mixture was stirred to room temperature over 2 h. Solvents were then removed by means of a rotary evaporator. The residue was taken up into water (50 mL) and ethyl acetate (50 mL) and the resulting mixture stirred to ice temperature. It was then acidified to pH 2−3 with 1 M $KHSO_4$ and the ethyl acetate layer removed. The aqueous layer was extracted twice with 40 mL portions of ethyl acetate, and the combined ethyl acetate extracts were dried over Na_2SO_4 and evaporated to dryness. The solid product (7.7 g) was characterized as *N*-Boc-[^2H]glycine by ^1H NMR.

N-Boc-[$^2H_2$]glycine (2.5 g, 18.5 mmol) was dissolved in [$^2H_4$]methanol (100 mL), and the solution was left at room temperature overnight. It was evaporated to dryness and dried further under oil pump vacuum overnight. Thioglycolic acid (1.42 mL, 20.4 mmol) was dissolved in [$^2H_4$]methanol under an atmosphere of N_2. After ca. 3 h, the methanol was removed by rotary evaporation and the residue dried under oil pump vacuum for 30 min. THF was dried over $LiAlH_4$ and distilled (100 mL) into a flask containing the *N*-Boc-[^2H]glycine. The latter dissolved on stirring the mixture which was then cooled to ice temperature. Carbonyl diimidazole (3.0 g, 18.5 mmol) was added and the reaction mixture stirred at 0 °C for 1 h. The deuterated thioglycolic acid, dissolved in dry THF (15 mL), was then added, and the final mixture stirred at 4 °C for 4 days. After completion of the reaction period, the solvent was removed by rotary evaporation. The residue was taken up into ethyl acetate (100 mL) and washed with 10% citric acid (2×100 mL) and water (2×100 mL). The dried (Na_2SO_4) solution was then evaporated to dryness, yielding an oil (3.36 g) whose ^1H NMR spectrum [([C^2HCl_3) δ 1.48 (9H, s, tBu), 3.71 (2H, s, CH_2), 5.08 (1H, br s, NH)] identified it as the required product, *N*-Boc-[$^2H_2$]glycylthioglycolic acid.

N-Boc-[$^2H_2$]glycylthioglycolic acid (1.29 g, 6.3 mmol) was dissolved in CH_2Cl_2 (4.5 mL) and the solution cooled to ice temperature with stirring. Trifluoroacetic acid (44.5 mL) was then added and the solution stirred to room temperature over 1 h. After removal of solvents by rotary evaporation, the product ([$^2H_2$]glycylthioglycolic acid) was dried under oil pump vacuum for 30 min. The residue was taken up into water (15 mL) containing $NaHCO_3$ (1.68 g, 20 mmol). A solution of *N*-phenylacetoxysuccinimide (1.4 g, 6.3 mmol) in dry acetonitrile (9 mL) was then added dropwise to the stirred aqueous solution at room temperature and the final mixture stirred for a further 1.5 h. After the acetonitrile was removed by rotary evaporation, the remaining aqueous solution was extracted with ethyl acetate (3×10 mL) and then acidified to pH 1.5 with 1 M HCl. The ensuring opaque solution was extracted with ethyl acetate (3×10 mL). The combined ethyl acetate extracts of the acidified solution were washed with water, dried over $MgSO_4$, and evaporated to dryness, yielding 0.55 g of crude product. This material was

purified by flash chromatography (3 × 10 cm silica gel column, eluted with ethyl acetate/hexane/acetic acid, 10/9/1 v/v) and by recrystallization from the same solvent mixture. The final yield was 160 mg, mp 105 °C. The product was characterized by its ^{1}H NMR [(C^2HCl_3) δ 3.69 (2H, s, Ph$\underline{CH_2}$), 3.73 (2H, s, CH_2S), 5.98 (1H, br s, NH), 7.2−7.5 (5H, m, ArH)] and mass spectrum [ESMS *m*/*e* 292.1]. Integration of the small glycyl methylene peak (δ 4.22) in the ^{1}H NMR spectrum indicated a protium content of 4%.

The dihydro analogue (phenylacetyl)glycylthioglycolic acid, **2a**, was prepared by direct condensation of (phenylacetyl)glycine and thioglycolic acid by a procedure directly analogous to that used previously for the synthesis of **3** (*14*). The product was recrystallized from ethyl acetate/hexane/acetic acid (10/9/1 v/v), and exhibited a melting point of 105−106 °C, an ESMS peak at *m*/*e* 290.1, and a ^{1}H NMR spectrum essentially identical to that of **2b** with the addition of a two-hydrogen doublet, representing the glycyl resonance, at δ 4.22.

*N,N′-Diacetyllysyl-[$^2H_2$]glycyl-*D*-alanine, **5b***. *N*-Boc-[$^2H_2$]-glycine, D-alanine *tert*-butyl ester hydrochloride (Bachem), and 1-hydroxybenzotriazole were deuterated at exchangeable positions by dissolution in [$^2H_4$]methanol as described above and dried prior to use. *N*-Boc-[$^2H_2$]glycine (0.97 g, 6.2 mmol) and 1-hydroxybenzotriazole (0.97 g, 6.2 mmol) were added to a suspension of D-alanine *tert*-butyl ester hydrochloride (1.3 g, 6.2 mmol) in dry THF (10 mL), and the mixture was stirred to ice temperature. Dicyclohexylcarbodiimide (1.55 g, 6.2 mmol) was then added, followed by *N*-ethylmorpholine (0.80 mL, 6.2 mmol), and the final mixture was stirred at 0 °C for 1 h. After further stirring of the reaction mixture to room temperature over 1 h, dicyclohexylurea was removed by filtration and the filtrate freed of solvent by rotary evaporation. The residue was taken up into ethyl acetate (30 mL) and the solution washed with saturated aqueous $NaHCO_3$ (2 × 20 mL), 10% citric acid (2 × 20 mL), saturated $NaHCO_3$ (20 mL), and water (20 mL). The ethyl acetate solution was then dried over $MgSO_4$ and the solvent removed by evaporation, leaving the product, *N*-Boc-[$^2H_2$]-glycyl-D-alanine *tert*-butyl ester, as an oil (1.61 g). The ^{1}H NMR spectrum supported the identification of the material as the required product: (C^2HCl_3) δ 1.38 (3H, d, J = 6, CH_3), 1.49 (18H, s, *t*-Bu_2), 4.47 (1H, quint, J = 6, CH), 5.09 (1H, br, NH), 6.60 (1H, br, NH).

[$^2H_2$]Glycyl-D-alanine was then obtained by removal of the *tert*-butyl protecting groups by trifluoroacetic acid treatment in an identical fashion to that described above in the preparation of glycylthioglycolic acid. Also as for the analogous reaction described above, [$^2H_2$]glycyl-D-alanine was reacted with *N,N′*-diBoc-lysine *N*-hydroxysuccinimide ester (Bachem) in aqueous acetonitrile to produce *N,N′*-diBoc-lysyl-[$^2H_2$]glycyl-D-alanine.

Finally, the *N,N′*-diacetyl compound was obtained by removal of the *t*-Boc groups from *N,N′*-diBoc-lysyl-[$^2H_2$]-glycyl-D-alanine by trifluoroacetic acid treatment as described above and acetylation of the consequent diamine by the procedure of Nieto and Perkins (*20*). Thus, 1.74 g (3.6 mmol) of the Boc-protected compound was treated with trifluoroacetic acid, and the product of the deprotection reaction was dissolved in a mixture of water (18.5 mL) and dioxane (18.5 mL). After the solution was cooled to 0 °C, triethylamine (4.07 mL, 29 mmol) was added with stirring followed by acetic anhydride (0.83 mL, 8.8 mmol) and the reaction allowed to proceed at 0 °C for 3 h. The reaction mixture was then evaporated to dryness under vacuum, and 30 mL of water was added and reevaporated. The residue was dried overnight under oil pump vacuum. The crude product was dissolved in the minimum volume of water (ca. 15 mL) and loaded onto a 24 × 1.8 cm Dowex 50 × 4-400 ion exchange column (H^+ form). The product, eluted with water, appeared in fractions (3 mL) 35−50. These were pooled and freeze-dried to give 0.82 g of the product. It was characterized by its ^{1}H NMR [($^2H_6$-DMSO) δ 1.27 (3H, d, J = 7.3, CH_3), 1.2−1.7 (6H, m, $(CH_2)_3$), 1.79 (3H, s, CH_3CO), 1.85 (3H, s, CH_3CO), 3.00 (2H, quart, J = 6, AcNH$\underline{CH_2}$), 4.17 (1H, quint, J = 6, $\underline{CH}CH_3$), 4.22 (1H, quart, J = 7.3, $\underline{CH}CH_2$), 7.79 (1H, br, NH), 8.04 (1H, br, NH), 8.07 (1H, br, NH), 8.16 (1H, br, NH), 12.53 (1H, br, CO_2H)] and mass spectrum [ESMS *m*/*e* 361.2]. Integration of the small glycylmethylene peak (δ 3.6) in the ^{1}H NMR spectrum indicated a protium content of ≤1%.

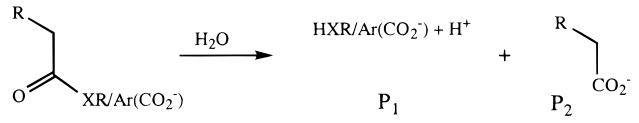
The dihydro analogue **5a** was prepared in an identical fashion from di-Boc-lysine *N*-hydroxysuccinimide ester and commercial glycyl-D-alanine (Bachem). The product exhibited a ^{1}H NMR spectrum essentially identical to that of **5b** with the addition of a two-hydrogen double AB quartet at δ 3.51, 3.74 (J = 6.3, 17.4), and a ESMS peak at *m*/*e* 359.2.

Steady-State Kinetic Methods. These were essentially as described in detail previously (*12*, *13*). The enzyme-catalyzed reactions of **1**−**7** were spectrophotometrically monitored at 300 nm ($\Delta\epsilon$ = 950 M^{-1} cm^{-1}) or 290 nm ($\Delta\epsilon$ = 1760 M^{-1} cm^{-1}), 280 nm ($\Delta\epsilon$ = 52 M^{-1} cm^{-1}), 350 nm ($\Delta\epsilon$ = 2.74 × 10^3 M^{-1} cm^{-1}), 350 nm ($\Delta\epsilon$ = 2.74 × 10^3 M^{-1} cm^{-1}), 238 nm ($\Delta\epsilon$ = 27 M^{-1} cm^{-1}), 238 nm ($\Delta\epsilon$ = 27 M^{-1} cm^{-1}), and 230 nm (695 M^{-1} cm^{-1}), respectively. The hydrolysis of **3** and **4** was monitored by release of 4-thiopyridone from 4,4′-dipyridyl disulfide (1 mM) in the presence of 0.1 mM EDTA (*11*). All enzyme kinetic experiments were performed at 25 °C in 20 mM MOPS buffer at pH 7.5, unless otherwise stated. The K_m value for **5** was obtained from measurements of the extent of inhibition by **5** of the hydrolysis of **1**.

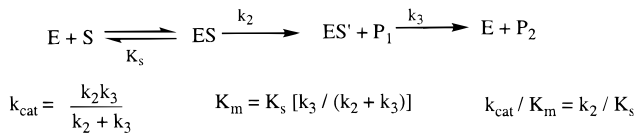
Methanolysis and aminolysis kinetics were also performed as previously described (*13*, *16*). Methanol concentrations of up to 3.0 and 1.5 M were employed for the P99 β-lactamase and the R61 DD-peptidase, respectively. Reactions of **1** (5 mM), **2a** (25 mM), **3** (9.9 mM), and **6** (20 mM) were studied in the presence of D-phenylalanine and/or D-leucine (0−30 mM).

Kinetic Isotope Effects. These were determined from spectrophotometrically determined reaction rates, largely as described in detail in a previous paper (*12*). The hydrolysis of **2**−**4** in these determinations was monitored by release of 4-thiopyridone from 4,4′-dipyridyl disulfide as described above. This procedure was found to give better precision than did direct observation of the thio ester absorption. The β-secondary kinetic isotope effects for hydrolysis of **5** by the R61 DD-peptidase were obtained from single-point determinations of reaction progress at 5−10% of completion. Thus, reaction mixtures containing enzyme (1.9 μM) and substrate (1.0 mM) were quenched at this stage (ca. 6 h) by immersion in boiling water for 2 min. Product D-alanine was then determined by a colorimetric method (*21*). The kinetic isotope effect was then obtained directly from the product ratio.

Scheme 1



Scheme 2



The β-secondary kinetic isotope effect for the alkaline hydrolysis of **2** was determined from pseudo-first-order rate constants obtained in 10 mM sodium hydroxide solution (μ = 1.0, KCl). The reactions were monitored spectrophotometrically at 250 nm.

*pH−Rate Profiles for the P99 β-Lactamase-Catalyzed Hydrolysis of **1** and **2**.* Spectrophotometric rate constants under *V/K* conditions ($\leq 0.1K_m$) were determined in a mixed buffer containing 20 mM each of pyridine, MES, MOPS, HEPES, and AMPSO. An ionic strength of 1.0 M was maintained with NaCl. A classical bell-shaped curve (one acid, one base) was fitted to the profiles by means of a nonlinear least-squares fitting program.

RESULTS AND DISCUSSION

The compounds **1**−**7** are substrates of both the *Streptomyces* R61 DD-peptidase and the *Enterobacter cloacae* P99 β-lactamase. Both enzymes catalyze the hydrolysis of these compounds, releasing the C-terminal leaving group (Scheme 1). Steady-state parameters for the turnover of **1** − **7** by these enzymes are shown in Table 1. Data are not shown for peptides **5** and **6** with the β-lactamase since these reactions were too slow for their rates to be determined with precision; the acylaziridine **7**, however, is both a peptide and a β-lactamase substrate (*16*). It will be assumed that these enzyme-catalyzed hydrolyses proceed by way of the reaction Scheme 2, where ES′ represents the covalent acyl-enzyme intermediate known to be formed with both of these enzymes (*7*, *22*−*24*). Most of the trends in the data of Table 1 have been previously noted (*11*, *16*).

It was important to determine for many of the substrates whether acylation of the enzyme or deacylation is rate-determining at saturation, i.e., whether k_{cat} is equal to, or more closely approximating, k_2 or k_3. This point can be decided for these enzymes by application of alternative nucleophiles, typically methanol or D-amino acids (*13*, *25*). If $k_{cat} = k_3$, the observed reaction rate at high substrate concentration should increase with nucleophile concentration, whereas if $k_{cat} = k_2$, this should not be true. The information available on this point is also presented in Table 1. As part of the present work, employment of the nucleophile D-phenylalanine has established (data not shown) that $k_{cat} = k_3$ for the R61 DD-peptidase-catalyzed hydrolysis of **1** and **2**. Experiments with D-leucine and the DD-peptidase with peptide **6** established that $k_{cat} = k_2$ in this case, a conclusion in accord with previous experiments (*26*). Finally, studies of catalysis by the P99 β-lactamase in the presence of methanol indicated that k_{cat} is more closely represented by k_2 than by k_3 for the substrate **2** (see further discussion of this case below, however). Other assignments presented in Table 1 arise from previously reported experiments (*13*, *16*).

It is noticeable that in the hydrolysis of **2** by the P99 β-lactamase neither k_2 nor k_3 solely determines k_{cat} (k_{cat} for **2** is 54 s^{-1} whereas k_3 is believed from k_{cat} for **1** to be 125 s^{-1}). A calculation employing the expression for k_{cat} in Scheme 2 yielded a value of k_2 for **2** of 95 s^{-1}. A similar caveat applies to **3**, but the difference between k_{cat} and k_2 in this case is negligible for the present purposes.

β-Secondary Kinetic Isotope Effects. As described in the introduction, these would be anticipated to be inverse for an acyl-transfer reaction, the actual magnitude providing an estimate of the degree of loss of hyperconjugation of the β-hydrogen atoms in the transition state and hence of the degree of its tetrahedral character. For example, the β-secondary kinetic isotope on alkaline hydrolysis of **2** was determined to be 0.922 ± 0.011. This value is very close to that previously obtained for **1** (*12*) and indicates that the transition state for this reaction closely resembles the tetrahedral intermediate (*12*).

The β-secondary kinetic isotope effects obtained for the enzyme-catalyzed hydrolyses of compounds **1**, **2**, and **5** are given in Table 2. Very similar transition states with respect to tetrahedral character were thus indicated for acylation (*V/K*) of the enzymes by the thioldepsipeptide **2** and of the R61 DD-peptidase by the peptide **5**. The reference ground state for these transition states is free enzyme and free substrate so these results can be directly compared with those for alkaline hydrolysis. Such a comparison suggests a less tetrahedral structure for the transition state of these enzyme-

Table 1: Steady-State Parameters for Hydrolysis of **1**−**7** by the R61 DD-Peptidase and the P99 β-Lactamase[a]

	enzyme					
	R61			P99		
substrate	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})
1	1.51 ± 0.09[g]	0.76 ± 0.11	2.0×10^3	125[b,g]	0.23[b]	5.43×10^5 [b]
2	2.16 ± 0.14[g]	1.45 ± 0.26	1.49×10^3	54 ± 2[h]	8.0 ± 0.8	6.71×10^3
3	3.28[c,g]	0.57[c]	5.8×10^3 [c]	22.3[c,h]	3.3[c]	6.7×10^3 [c]
4	5.93[c,d]	1.54[c,d]	3.9×10^3 [c,d]	5.08[c]	6.65[c]	7.6×10^2 [c]
5	0.33 ± 0.01[h]	17.8 ± 2.9	18.5	−	−	−
6	34.5 ± 2.5[h]	9.8 ± 1.5	3.52×10^3	−	−	−
7	4.5[e,f,g]	7.6[e,f]	6.0×10^2 [e,f]	130[e,g]	3.4[e]	3.9×10^4 [e]

[a] 20 mM MOPS, pH 7.5, 25 °C unless otherwise noted. [b] Data from Adediran et al. (*12*). [c] Data from Xu et al. (*14*). [d] 20 mM phosphate buffer, pH 7.0, 37 °C. [e] Data from Murphy and Pratt (*16*). [f] 0.1 M phosphate buffer, pH 7.0, 37 °C. [g] $k_{cat} \approx k_3$ (see text). [h] $k_{cat} \approx k_2$ (see text).

Table 2: β-Secondary Kinetic Isotope Effects[a]

	enzyme			
	R61 DD-peptidase		P99 β-lactamase	
substrate	$^{\beta\text{-D}}V/K$	$^{\beta\text{-D}}V$	$^{\beta\text{-D}}V/K$	$^{\beta\text{-D}}V$
1	1.040 ± 0.005 (4) 1.013 ± 0.020 (4)	0.947 ± 0.026 (7) 0.949 ± 0.026 (7)	1.002 ± 0.012[b]	0.994 ± 0.026[b]
2	0.964 ± 0.018 (8) 0.971 ± 0.020 (6) 0.952 ± 0.023 (8)	0.959 ± 0.026 (8)	0.952 ± 0.016 (6) 0.970 ± 0.018 (8)	1.056 ± 0.023 (7) 1.053 ± 0.028 (7)
5	0.959 ± 0.013 (5) 0.960 ± 0.021 (7)	*c*	*c*	*c*

[a] The values given are means with standard deviations; the number of pairs of kinetic runs are given in parentheses. The values for a given parameter, enzyme and substrate on separate lines, represent separate experiments with separately prepared enzyme and substrate solutions. [b] Data from Adediran et al. (*12*). [c] Not determined.

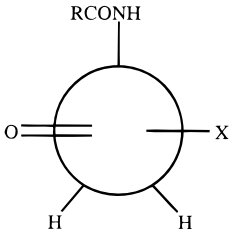
catalyzed reactions although a number of factors involving the nature of the binding site and the conformation of the bound substrate may also lead to a less inverse isotope effect (*12*). In particular, as noted in a recent paper by Hess et al. (*27*), one such factor is electrophilic catalysis provided by the oxyanion hole.

The measurement of $^{\beta\text{-D}}V/K$ for **2** and the P99 β-lactamase is complemented by the measurement of $^{\beta\text{-D}}V$ for this combination of enzyme and substrate. As noted above, k_{cat} for **2** with this enzyme is a composite quantity (Scheme 1), and thus $^{\beta\text{-D}}k_2$ must be calculated from eq 1. Given that $k_3 = 125\ s^{-1}$ and $^{\beta\text{-D}}k_3 = 0.994$ (data from **1** and the P99 β-lactamase), $^{\beta\text{-D}}k_2$ can be calculated to be 1.095 ± 0.040:

$$^{\beta\text{-D}}V = {}^{\beta\text{-D}}\left(\frac{k_2k_3}{k_2 + k_3}\right) \quad (1)$$

If $^{\beta\text{-D}}(k_2/K_s) = {}^{\beta\text{-D}}(V/K) = 0.96$ and $^{\beta\text{-D}}k_2 = 1.095$, then $^{\beta\text{-D}}(1/K_s) = 0.88$. The results thus suggest a decrease in hyperconjugation on binding and an increase on chemical reaction. This appears counterintuitive but can be rationalized. As previously discussed with respect to **1**, the most favorable ground state conformation of these depsipeptides in solution resembles **8** or **9** (*12*).

If, however, the bound conformation resembled **10**, the extent of hyperconjungation of the β-CH



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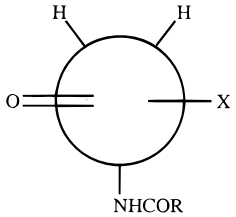
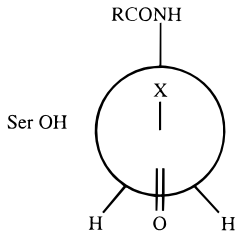


9

[proportional to $\cos^2\theta$, where θ is the angle between the β-CH bond and the carbonyl carbon p orbital (*28*)] would in fact decrease on binding and thus $^{\beta\text{-D}}(1/K_s)$ would be inverse, as observed. A late transition state such as **11**, involving breakdown of the tetrahedral intermediate (see below), could then give rise to the increase in hyperconjugation seen in the acylation step ($1 \times \cos^2 30° > 2 \times \cos^2 60°$) and hence to the normal isotope effect on k_2. It is interesting that this complexity is



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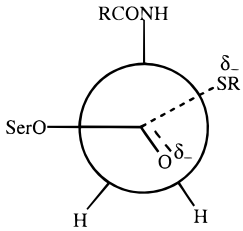

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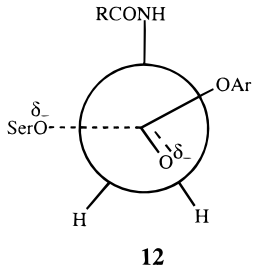
concealed within $^{\beta\text{-D}}V/K$ and only evident if either $^{\beta\text{-D}}k_2$ or $^{\beta\text{-D}}(1/K_s)$ can be separately determined. It might therefore be true of some or all of the other substrates or with the DD-peptidase in cases where such separate determinations could not be made.

Acylation of the two enzymes by **1** also leads to isotope effects and thus transition states similar to each other. The slightly normal isotope effects, however, suggest that the transition states are rather different from those derived from **2** and **5**. This situation for **1** and the P99 β-lactamase has been discussed previously in terms of an additional nonchemical step prior to the acylation reaction (*12*). In terms of Scheme 2 as it stands however, the results might be accommodated, by analogy to the proposal for **2** above, in terms of a transition state such as **12**



12

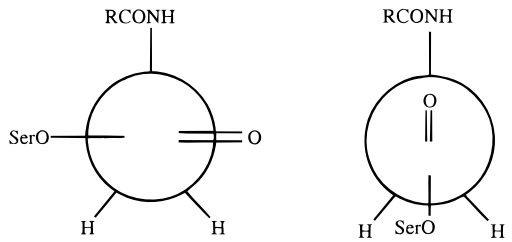
where a net decrease of hyperconjugation similar to that in **11** might be achieved. To achieve a slightly normal value of $^{\beta\text{-D}}V/K$ rather than the inverse effect as found for **2**, **12** must be somewhat further from tetrahedral than is **11**. A different degree of electrophilic catalysis by the oxyanion hole may also be a factor. The transition state shown is one appropriate to formation of the tetrahedral intermediate rather than breakdown because of the small leaving group effects observed for aryl ester substrates of the P99 β-lactamase (*13*).

Table 3: Solvent Deuterium Kinetic Isotope Effects[a]

substrate	enzyme			
	R61 DD-peptidase		P99 β-lactamase	
	$^{D_2O}V/K$	^{D_2O}V	$^{D_2O}V/K$	^{D_2O}V
1	0.85 ± 0.06 (6)	2.56 ± 0.19 (6)	0.82[b]	1.44[b]
2	1.04 ± 0.04 (6)	2.24 ± 0.14 (6)	1.01 ± 0.02 (6)	1.17 ± 0.04 (6)
3	*c*	*c*	1.07 ± 0.02 (6)	1.16 ± 0.04 (6)
4	0.87 ± 0.03 (9)	*c*	1.12 ± 0.07 (6)	*c*
5	0.94 ± 0.06 (4)	*c*	*c*	*c*
6	0.89 ± 0.06 (14)	2.70 ± 0.16 (10)	*c*	*c*
7	1.17 ± 0.04 (9)	*c*	1.09 ± 0.06 (9)	*c*

[a] The values given are means with standard deviations; the number of pairs of kinetic runs is given in parentheses. [b] Data from Adediran et al. (*12*). [c] Not determined.

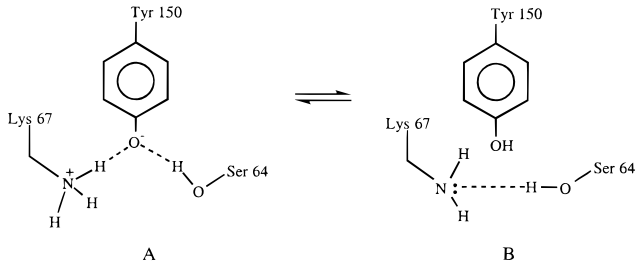
β-Secondary kinetic isotope effects on the deacylation step are available ($^{\beta\text{-D}}V$) for the R61 DD-peptidase and substrates **1** and **2**. These suggest typical acyl transfer transition states with considerable tetrahedral character. The isotope effects should, in principle, if Scheme 2 obtains, be identical, since the same acyl-enzyme would be expected from **1** and **2**. Within experimental uncertainty, the isotope effects are in gratifying agreement with this expectation. The $^{\beta\text{-D}}V$ effect on **1** with the P99 β-lactamase also reflects a deacylation reaction. Here, however, as noted prevously (*12*), the isotope effect is very close to unity. This result was previously interpreted (*12*) in terms of the conformation of the acyl-enzyme which must resemble **13** (readily accessible via **11**) where the β-hydrogens only weakly hyperconjugate. A combination of this interpretation with the present results with the DD-peptidase leads to the conclusion that the (phenylacetyl)glycyl derivative of the latter enzyme must have a significantly different conformation than that of the P99 β-lactamase: a more strongly hyperconjugating conformation such as **14** might obtain in the DD-peptidase, for example.



Solvent Kinetic Isotope Effects. Table 3 contains the solvent deuterium kinetic isotope effects determined in the current group of experiments. The most striking result evident from Table 3 is that the *V/K* values are uniformly very close to unity, and even slightly inverse in several cases. At face value, this suggests the complete absence of proton motion in the acylation (*V/K*) transition state for both enzymes. It is also common to both side chains, phenylacetyl and *N*,*N*′-diacetyllysyl. This is very unusual for the transition state of alcoholysis of an acyl derivative, where one would expect general base catalysis of nucleophilic attack in the formation of the tetrahedral intermediate and general acid catalysis of leaving group departure in breakdown of the tetrahedral intermediate (*29*, *30*). Although there may be some debate over whether thiolate or phenolate leaving groups absolutely require protonation during departure (see below), there is little uncertainty when the leaving group is an amine as in **5**−**7**. Solvent kinetic isotope effects of similar magnitude have been observed for hydrolysis of β-lactams by the P99 β-lactamase where the leaving group is also an amine (*12*, *31*). In contrast, definitively normal (1.4−3.3) solvent isotope effects have been routinely observed for k_{cat}/K_m ($=k_2/K_s$) values with serine proteinases (*19*, *32*, *33*), where general acid/base catalysis is very well-established. The total absence of such catalysis in acylation of β-lactam-recognizing enzymes would be quite surprising. Most candidate mechanisms for these particular enzymes certainly involve such catalysis (*23*, *24*, *34*).

Insight into the dilemma posed by the solvent isotope effects on *V/K* came from consideration of the corresponding isotope effect on *V* for peptide **6** and the R61 DD-peptidase. This value of 2.70 is clearly evocative of general acid/base catalysis as expected for amide hydrolysis and, furthermore, corresponds to the rate constant k_2 and its corresponding transition state. The result is also in accord with the $^{\beta\text{-D}}V$ measurement on peptide **5** where acyl-transfer chemistry is evident (see β-secondary isotope effects above). Returning to the solvent isotope effect, however, if $^{D_2O}k_2 = 2.70$ and $^{D_2O}V/K = {}^{D_2O}k_2/K_s = 0.89$, then $^{D_2O}(1/K_s) = 0.33$. The two extreme interpretations of a solvent isotope effect of 0.33 on substrate binding are, in terms of fractionation factors (*35*), on the one hand, $\phi^{E+S} = 1$, $\phi^{ES} = 3.0$, an unlikely scenario since a fractionation factor of 3.0 is difficult to imagine (*35*−*37*), and, on the other, $\phi^{E+S} = 0.33$, $\phi^{ES} = 1$, the more likely possibility. It is difficult to see a fractionation factor of 0.33 for any exchangeable proton in free substrate, particularly since **1**−**7** differ significantly from each other in structure. Hence, a more likely interpretation of the latter extreme would be that of $\phi^E = 0.33$. This result agrees rather nicely with a result obtained by Page et al. (*31*) with the P99 β-lactamase. They determined pH-rate (*V/K*) profiles for both the hydrolysis and the methanolysis of benzylpenicillin, and found that ΔpK_a ($=pK_a^{D_2O} - pK_a^{H_2O}$) had the unusually large value of 0.85, rather than the expected value of 0.5 (*35*−*37*), and suggested that the likely reason was in fact a low fractionation factor in the free enzyme for protons involved in catalysis. A calculation based on the ΔpK_a value of 0.85 gives a ϕ^E value of 0.43 if it is assumed that ϕ^{EH}, the fractionation factor of the revelant protons in the low-pH and inactive form of the enzyme, is 1.0. These results then suggest that in the active form of the enzyme free in solution at neutral pH, active site protons in both the R61 DD-peptidase and the P99 β-lactamase may have a net

Scheme 3



fractionation factor of around 0.4. This situation would thus affect the solvent isotope effects of all substrates. Certainly, $^{D_2O}V/K$ for all of **1**−**7**, encompassing a wide range of different leaving groups as well as two side chains, *N*-phenylacetyl to *N*,*N*′-diacetyllysyl, did not substantially change for either enzyme, and the values for β-lactam substrates are similar (*12*, *31*). Page et al. (*31*) have suggested that the protons involved are those of the ammonium ions of Lys 315 and Lys 67 which are believed to stabilize, by electrostatic interaction and hydrogen-bonding, Tyr 150 in the phenoxide form (*24*, *34*, *38*). Thus, ionized Tyr 150 can then participate as a general base in catalysis (Scheme 3A). This proposal seems reasonable, at least with respect to Lys 67 and Tyr 150, since direct analogues of these are found at the R61 DD-peptidase active site (*5*).

The other interesting consequence of this analysis is of course the finding that $\phi^{ES} = 1$ for the R61 DD-peptidase and **6**. This appears to imply that the low fractionation factors of the active site protons are lost on peptide binding, presumably through internal rearrangement. One possibility would be conversion to the tautomeric form B (Scheme 3) where the protons are arranged such that the Lys 67 amine rather than the Tyr 150 phenoxide functions as the general base in catalysis. This role of Lys 67 in R61 DD-peptidase catalysis has been tentatively suggested by Jamin et al. (*22*) although completely unambiguous methods for distinguishing A and B as the reactive species after substrate binding have been difficult to achieve.

Given these conclusions, ^{D_2O}V for **2** (and **3**) with the P99 β-lactamase should be considered. Application of the solvent isotope analogue of eq 1 to **2**, employing $^{D_2O}V = 1.17$ and $^{D_2O}k_3 = 1.44$ (from ^{D_2O}V of **1**), yields $^{D_2O}k_2 = 0.93$, a value very different from that of **6** and one suggesting little proton transfer in the acylation transition state. Further, since $^{D_2O}V/K = {}^{D_2O}k_2/K_s = 1.01$ (taking the value for **2**), then $^{D_2O}(1/K_s) = 1.1$, and, since $\phi^E = 0.4$, it follows that $\phi^{ES} = 0.36$. This implies no active site proton rearrangement on substrate binding, and perhaps therefore that the reactive tautomer in this case is A (Scheme 3). It is not clear at this stage whether the difference in this regard observed between the DD-peptidase and the β-lactamase is general or only specific to the substrates employed, viz., the peptide **6** and the thiodepsipeptides **2** and **3** for the peptidase and β-lactamase, respectively. The proton configuration in the noncovalent complex may in fact be substrate- and enzyme-dependent.

The value of $^{D_2O}k_2$ obtained for **2** and **3** with the P99 β-lactamase gives no suggestion of proton transfer accompanying acylation. This might be possible if a transition state such as **11** obtained, and if the leaving group did not require protonation as part of turnover. This is quite likely for departure of a thiolate, as previously discussed (*14*).

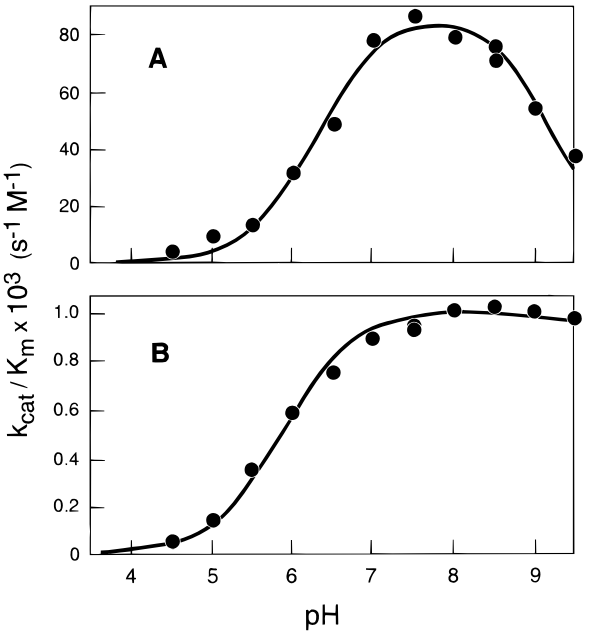


FIGURE 1: pH−rate (k_{cat}/K_m) profiles for hydrolysis of the depsipeptide **1** (A) and the thiodepsipeptide **2** (B).

Further evidence on this point is available from Figure 1 which shows the pH−*V/K* profile for the P99 β-lactamase-catalyzed hydrolysis of **1** (A) and **2** (B). The solid line on the profile for **1** is generated by a scheme where activity is controlled by two functional groups: a base, whose conjugate acid has a pK_a of 6.27 ± 0.11, and an acid of pK_a 9.26 ± 0.12. This profile is very similar to that obtained under the same conditions in this laboratory for a cephalosporin substrate where pK_as of 5.92 ± 0.16 and 9.26 ± 0.35 were obtained (*39*). In contrast, the profile for **2** shows the requirement for the base, with conjugate acid pK_a of 5.85 ± 0.09, but not for the acid; the data of Figure 1 require that any acid pK_a for **2** must be greater than or equal to 10.8 (the solid line shown represents that for a pK_a of 10.8). This suggests that an acid functionality is required for hydrolysis of β-lactams and depsipeptides such as **1** but not for thioldepsipeptides such as **2**. Since the profile shown is that of k_{cat}/K_m, and hence of k_2/K_s according to Scheme 2, the acid is specifically not required for acylation of the β-lactamase by **2**. The k_{cat}/K_m pH−rate profile for the hydrolysis of peptide **6** by the R61 peptidase has, as expected for an amide leaving group, requiring protonation, a descending limb with pK_a of 9.47 (*26*).

The requirement of acid catalysis for **1**, in its turnover by the β-lactamase (as indicated by Figure 1A), as well as for peptide **6** (and presumably **5** and **7** as well) by the DD-peptidase, suggests that $^{D_2O}k_2$ would be significantly normal for **1** and hence, given $^{D_2O}V/K = 0.82$ and $\phi^E = 0.4$, that ϕ^{ES} would be close to unity. Thus, using the rationale introduced above, one would argue that the tautomeric equilibrium of Scheme 3 for β-lactamase-bound **1** would also lie in favor of B. This argument is not quite tight, however, and remains an interesting speculation to be tested further. One further experimental result which also distinguishes the *V/K* transition states for **1** and **2** is the effect of 2 M ammonium sulfate on them. The ratio of k_{cat}/K_m in buffer to that in buffer also containing 2 M ammonium sulfate is 3.60 ± 0.35 and 5.20 ± 0.30 for **1** and **2**, respectively. These are

sufficiently different to reflect some significant transition state difference, perhaps that of proton distribution as discussed above.

Finally, there remains the solvent isotope effects on deacylation (k_3). Deacylation of the (phenylacetyl)glycyl derivatives of the R61 DD-peptidase, arising from its reaction with **1** and **2**, shows a significant effect clearly indicating the presence of proton transfer in the transition state and thus presumably general acid/base catalysis. This result is in good accord with the $^{\beta\text{-D}}V$ isotope effects (Table 2) which indicate acyl-transfer chemistry. Much less proton motion is indicated by ^{D_2O}V for deacylation of the (phenylacetyl)-glycyl derivative of the P99 β-lactamase, derived from its reaction with **1**. This may reflect the effects of a different orientation of the substrate with respect to the active site functional groups, as suggested above from interpretation of $^{\beta\text{-D}}V$.

Concluding Discussion. The results described above have been interpreted in terms of the simplest possible reaction scheme (Scheme 2), with enzyme acylation and deacylation steps following substrate binding. Although it is possible that there are other kinetically significant steps, there is no unequivocal evidence for them at present with the substrates examined in this work. The results showed much that is common in the transition states of reactions catalyzed by the R61 DD-peptidase and the P99 β-lactamase, as might be anticipated in view of the similarities in their active site structures (*5*−*7*). On the other hand, differences were also observed. The generality of some of these observations to the specificity of the enzyme involved, DD-peptidase or β-lactamase, however, is not yet clear.

Striking among the β-secondary isotope effects was the absence of a distinctly inverse $^{\beta\text{-D}}V/K$ value in both enzymes for the depsipeptide **1**, which must reflect some gain in β-CH hyperconjugation in the transition state over the level present in **1** in free solution. This can be rationalized in terms of a specific conformation of **1** in the transition state, such as that depicted as **12**. Also striking was the different $^{\beta\text{-D}}V$ values for **1** between the two enzymes, which was interpreted in terms of different acyl-enzyme conformations, **13** and **14**. With regard to the latter point, inspection of crystal structures did not immediately reveal to us the structural basis of this difference. Although Ser 62 O_γ, the primary nucleophile of the R61 DD-peptidase active site, appears rather differently placed with respect to the auxiliary functional groups Lys 62 N_ϵ and Tyr 159 OH than the corresponding residues of the P99 β-lactamase when the crystal structures of the free enzymes are considered (*5*, *6*), the former Ser O_γ moves into a very similar position to the latter when an acyl-enzyme structure is considered (*24*). Only a simple $C_\alpha-C_\beta$ rotation seems necessary for this interconversion. Ser 64 O_γ of the P99 enzyme is found in essentially the same position in a transition state analogue complex as in the free enzyme (*34*). The difference in transition state structure detected by the secondary isotope effects may, however, be related to the subtle functional distinction between these active sites leading one enzyme to be a DD-peptidase and the other a β-lactamase (*11*).

Although the $^{D_2O}V/K$ isotope effects suggest, counterintuitively, very little proton transfer in the acylation transition states for **1**−**7** with both enzymes, a closer analysis suggests that in the case of ester and amide substrates at least, this conclusion is not correct because of the effects of proton rearrangement accompanying substrate binding—the decrease in proton fractionation factors in the transition state of the acylation reaction is offset by an increase during the binding step. The latter explanation might also obtain with class A β-lactamases where very small normal or inverse $^{D_2O}V/K$ isotope effects have also been observed with **1** and β-lactams (*12*). This situation may be generally true for all β-lactam-recognizing enzymes in their reactions with peptides and β-lactams since they all have very similar active site components to those found in class A and class C β-lactamases (*40*). In the case of the P99 β-lactamase and the thiolester substrates **2** and **3**, a change in proton fractionation factors does not seem to occur on substrate binding, and hence the conclusion of little or no proton transfer in the *V/K* transition state appears to be correct in this case. This could be interpreted in terms of a transition state involving breakdown of the acylation tetrahedral intermediate where the departing thiolate leaving group does not require protonation. The latter proposition was supported by the *V/K* pH−rate profile for hydrolysis of **2** by the P99 β-lactamase (Figure 1).

Solvent isotope effects indicate the presence of proton transfer in the transition states of the deacylation steps of both enzymes, although the degree of proton transfer may be less for the β-lactamase. This general conclusion has also been reached for class A β-lactamases on reaction with both ester and β-lactam substrates (*12*). General acid/base catalysis therefore seems likely to be generally present in the reaction of these substrates with both DD-peptidases and β-lactamases, as most proposed mechanisms suggest.

The evolutionary process leading from a DD-peptidase to a β-lactamase seems to have involved little change in transition states except in their orientation with respect to active site functional groups and thus in the conformation of the bound substrates. This conclusion supports an earlier proposition (*16*) that a change in the shape of the active site, accommodating to that of the substrate, is one important difference between the two classes of enzymes. The structural differences involved in this change of shape have been discussed by Knox et al. (*7*).

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