Anomalous pH Dependence of the Reactions of Carbenicillin and Sulbenicillin with *Bacillus cereus* β-Lactamase I. Influence of the α-Substituent Charge on the Kinetic Parameters†

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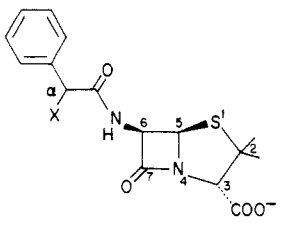
ABSTRACT: The pH dependence of k_{cat} for the *Bacillus cereus* β-lactamase I catalyzed hydrolysis of carbenicillin (VI), which differs from benzylpenicillin (I) in having a carboxylic moiety α to the phenyl ring, exhibits a profile consistent with a model in which the α-COOH and α-COO^- forms of the ES complex turn over with respective rate constants of 2152 s^{-1} and 384 s^{-1}. The pK_a(app) for the α-COOH is shifted from 3.2 in solution to 6.1 in the ES complex. The normalized k_{cat}/K_m vs. pH profile for VI is not superimposable on that of I, indicating that both the neutral and anionic forms of the carboxyl moiety of VI combine with the enzyme to give the first irreversibly formed complex, presumably the acyl-enzyme. Quantitative accord with the kinetic data is achieved only through fitting to a model where kinetically significant proton transfer in the ES complex is permitted. The second-order rate constants for the reaction of the enzyme with the α-COOH and α-COO^- forms of VI are 2.2×10^8 M^{-1} s^{-1} and 3.8×10^6 M^{-1} s^{-1}, respectively. The high value for the α-COOH form suggests that this reaction may be in part diffusion controlled. This conjecture is borne out by the observation that the sensitivity of k_{cat}/K_m to η_{rel} decreases with increasing pH for VI, whereas this sensitivity is pH independent for I. These conclusions are further supported by the results of a kinetic investigation of the pH dependence of sulbenicillin (VII) where an α-SO_3H replaces the α-COOH of VI. The strongly acidic sulfonic acid moiety of VII is fully ionized throughout nearly the entire pH range of interest, and its kinetics, as a function of pH, are very similar to those observed and calculated for the α-COO^- form of VI. Solvent deuterium kinetic isotope effects are reported for k_{cat} and k_{cat}/K_m for both VI and VII.

Several of the so-called "broad-spectrum" penicillins are



X	substrate
$-CO_2^-$	carbenicillin, VI
$-SO_3^-$	sulbenicillin, VII
$-CONH_2$	carbenicillin α-amide, VIII
$-NH_3^+$	ampicillin, IX

derivatives of benzylpenicillin (I) that are substituted with polar groups at the α-carbon of the phenylacetamido side chain (Price, 1969). The presence of such a substituent changes not only the range of bacteria susceptible to the killing action of a penicillin but also the kinetic parameters for the catalytic hydrolysis of the penicillin by bacterial β-lactamases (Price, 1969; Johnson et al., 1975; Waley, 1975; Davies et al., 1974; Hou & Poole, 1973; Richmond & Sykes, 1973). Waley (1975) has interpreted the change in the k_{cat} vs. pH profile of ampicillin (IX), with respect to that of I, in terms of the influence of the ionization of the α-amino group of ampicillin. The results reported here demonstrate that both the α-COOH and the α-COO^- forms of VI are hydrolyzed by the *Bacillus cereus* β-lactamase I, but with significantly different values of k_{cat} and k_{cat}/K_m.

Materials and Methods

Materials. The preparation and general assay procedures for *B. cereus* β-lactamase I are described in Hardy & Kirsch (1984a,b). The disodium salt of carbenicillin (VI)[1] was purchased from Sigma, and the monosodium salt was the generous gift of Beecham Laboratories. Experiments done with both salts of VI gave identical results. The disodium salt of sulbenicillin (VII) was donated by Takeda Industrial Chemicals. The α-amide of carbenicillin (VIII, ca. 70% pure) was provided as the monosodium salt by Gist-Brocades nv.

The buffers used were potassium acetate/sulfate, $I_c \sim 0.2$ (pH ≤5.6, pD ≤6.4) and potassium phosphate/pyrophosphate, $I_c = 0.2$ (pH ≥5.6, pD ≥ 6.0). Deuterated and sucrose-containing buffers were prepared as described previously (Hardy & Kirsch, 1984a,b).

Methods. The hydrolysis of VI was monitored at 235 or 242 nm, where the changes in molar absorbency accompanying hydrolysis (in H_2O and 0% sucrose) are 958 (SE = 12) (±12) and 534 (±15), respectively. The hydrolysis of VII was followed at 234 nm, where the change in molar absorbency upon hydrolysis is 1047 (48). The hydrolysis of VIII was monitored at 233 nm, where the change in molar absorbency accompanying hydrolysis is 796 (range = 42), based on 70% purity of the sample of this substrate used.

The disodium salt of VI (70-μmol samples) was titrated with a standardized solution of HCl (2.91 M) in 10 mL of 0.2 M KCl and 10 mL of 0.2 M KCl containing 32% w/v (ca. 28% w/w) sucrose, at 25 °C. Addition of sucrose does not substantially alter the ionization constants of VI since the titration curves in the presence and absence of sucrose were nearly superimposable.

Rate equations were derived by using the procedure of Cleland (1975), except for the analysis of the model shown in Scheme III, which was done by using the methods of King & Altman (1956) as modified by Cha (1968). The King–

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[1] Compounds I–V have been defined in the preceding papers (Hardy & Kirsch, 1984a,b).

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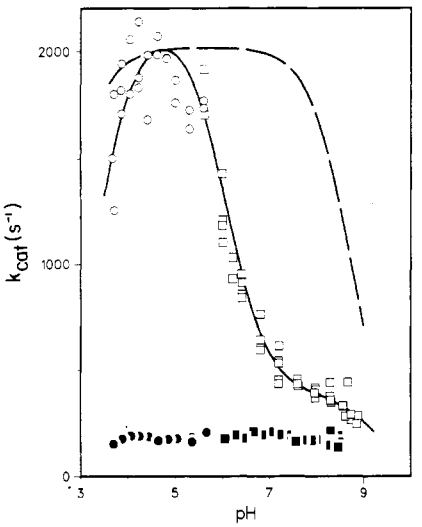
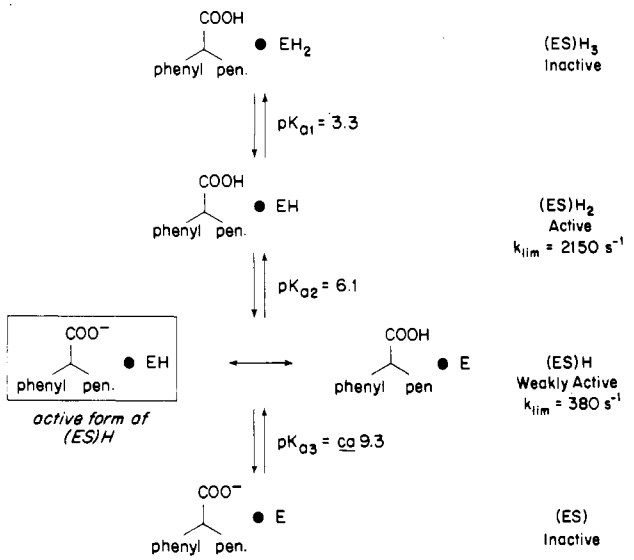


FIGURE 1: pH dependence of k_{cat} for the *B. cereus* β-lactamase I catalyzed hydrolysis of carbenicillin (VI, open symbols) and sulbenicillin (VII, solid symbols), in potassium acetate/sulfate (circles) and potassium phosphate/pyrophosphate (squares) buffers, I_c = 0.2, 25 °C. Component concentrations were as follows: [β-lactamase] = 2.9–33.1 nM, [VI] = 0.5–2.5 mM; [β-lactamase] = 13.4–29.6 nM, [VII] = 0.27–0.48 mM. Each data point is the value calculated from the complete time course of one kinetic run. The solid line is the theoretical curve for the mechanism shown in Scheme I, calculated from eq 1 by using the pK_a values listed in Table I for H_2O. The pH profile of k_{cat} for benzylpenicillin (I; Hardy & Kirsch, 1984b) is also shown (broken line) for comparison.

Scheme I



Altman patterns for this analysis were generated by using a Fortran program developed by Lam & Priest (1972), a listing of which was generously provided by the authors.

Results and Discussion

Dependence of k_{cat} on pH. The values of k_{cat} for the *B. cereus* β-lactamase I catalyzed hydrolyses of VI and VII are plotted as functions of pH in Figure 1. The value of k_{cat} for VI relative to that of benzylpenicillin (I) measured at pH 7.0 is similar to that reported for VI with *B. cereus* β-lactamase I (at pH 7.0, 30 °C) by Davies et al. (1974). The k_{cat} vs. pH profile for VI decreases above pH 5 and shows a pronounced "shoulder" above ca. pH 7. A comparison of the pH profile for VI with that for I [broken line in Figure 1; from Hardy & Kirsch (1984b)] suggests that there are two forms of ES complex formed with VI, differing by a single proton, which

Table I: Apparent pK_a Values for the pH Dependence of k_{cat} for the *B. cereus* β-Lactamase I Catalyzed Hydrolysis of Carbenicillin (VI)[a]

	pK_{a1} (SE)	pK_{a2} (SE)	pK_{a3} (SE)
H_2O	3.3 (0.1)	6.1 (0.1)	9.3 (0.5)
D_2O	3.9 (0.1)	6.6 (0.1)	9.7 (0.2)

[a] From nonlinear least-squares regression of the data on eq 1. Experimental conditions are given in the legend to Figure 1.

Table II: Effect of α-Substituent on k_{cat} for *B. cereus* β-Lactamase I Catalyzed Hydrolyses of α-Substituted Benzylpenicillins

compound	α-substituent	k_{cat} (s^{-1})[a]
IX	NH_3^+	8000[b]
VIII	$CONH_2$	4430[c] (±100)[d]
VI	COOH	2152[e] (62)[f]
I	H	2300[b]
		2000[g]
VI	COO^-	384[e] (51)[f]
VII	SO_3^-	197[h] (18)[f]

[a] Determined at 25 °C except as noted. [b] From Waley (1975); determined at 30 °C. [c] Average of two determinations, one each at pH 6.6 and pH 7.0 in potassium phosphate/pyrophosphate buffers, I_c = 0.2, with [β-lactamase] = 1.46 nM and [VIII] = 0.712 mM. Under these conditions, K_m for VIII is 0.25 (±0.01) mM. [d] Range/2. [e] From nonlinear least-squares regression of the data in Figure 1 on eq 1. [f] SE. [g] From Hardy & Kirsch (1984b). [h] Average of multiple determinations between pH 4 and pH 8.5, some of which are shown in Figure 1.

can undergo catalytic turnover with different rate constants. This hypothesis is diagramed in Scheme I. The pH dependence of k_{cat} for the mechanism shown in Scheme I is given by eq 1:

$$k_{cat} = \frac{[k_{(ES)H_2}]_{lim}}{H/K_{a1} + 1 + K_{a2}/H + K_{a2}K_{a3}/H^2} + \frac{[k_{(ES)H}]_{lim}}{H^2/K_{a1}K_{a2} + H/K_{a2} + 1 + K_{a3}/H} \quad (1)$$

The observed values for k_{cat} for VI in H_2O (Figure 1) and D_2O (Hardy, 1983) were fit by nonlinear least-squares regression to eq 1. The pK_a values obtained are given in Table I and the limiting rate constants in Table II. The solid line in Figure 1 represents the theoretical curve for VI calculated from eq 1 by using the optimized kinetic parameters.

Two inactive and two active protonic forms of the complex formed from VI and β-lactamase I immediately precede the overall rate-determining step in catalysis according to the model depicted in Scheme I. The values of the pK_as separating the inactive from the active forms, 3.3 (0.1) and 9.3 (0.5), are similar to the pK_a values characterizing the pH dependence of k_{cat} for I, 2.6 (0.3) and 8.7 (0.1) (Hardy & Kirsch, 1984b). The differences are barely larger than the estimated errors. If real, they may be due to the effects of the carboxylic acid of VI on pK_{a1} and of the corresponding carboxylate on pK_{a3}. The additional pK_a in the pH profile of VI (pK_{a2}) arises from a proton transfer that interconverts the two active forms of the enzyme–substrate complex. This additional ionization must be due to the α-carboxyl group of VI, which is the only structural difference between I and VI. The ensemble (ES)H includes the kinetically indistinguishable structure [the right half of (ES)H in Scheme I] in which the proton is on the carboxyl group of VI. By analogy to the pH dependence of

k_{cat} for I, the latter component of (ES)H would be inactive.

The active form $(ES)H_2$, in which the α-carboxyl group of substrate VI is protonated and thus uncharged, has a k_{cat} = 2150 s^{-1} (Table II), similar to that obtained with benzylpenicillin (2000 s^{-1}; Waley, 1975; Hardy & Kirsch, 1984b). This assignment is supported by the observed dependence of k_{cat} for VII, which bears the more acidic sulfonic acid in place of the carboxylic acid of VI. The former species should have its α-substituent fully ionized throughout the accessible pH range (but see below). Its pH-independent value of k_{cat} = 200 s^{-1} is close to that for the assigned anionic form of VI (Table II).

The important effect of the α-substituent on the value of k_{cat} is documented in Table II. Benzylpenicillins having neutral or cationic α-substituents show k_{cat} values of 2000 s^{-1} or greater, whereas the values of k_{cat} for substrates having anionic α-substituents are much lower. The trend shown by these figures is consistent with a model in which the α-substituent of substituted benzylpenicillins interacts with an hydrogen-bond acceptor at the active site of *B. cereus* β-lactamase I.

The apparent dissociation constant for the α-carboxyl group of VI in the enzyme–substrate complex (ca. 10^{-6}) is more than 2 orders of magnitude lower than the value of the dissociation constant for this group in the free substrate ($10^{-3.2}$). Such a large decrease in the acidity of a carboxyl group upon formation of an enzyme–substrate complex is not unprecedented; for example, the pK_a of Glu-35 of lysozyme increases from 6 to 8–8.5 upon binding glycol chitin (Parsons & Raftery, 1972). In the present case, however, the perturbed pK_a is apparently that of a *substrate* carboxyl group, a situation more closely analogous to that observed in the binding of benzyl succinate to carboxypeptidase A (Palmer et al., 1982). Although the pH dependence for inhibition of carboxypeptidase by benzyl succinate is consistent with exclusive binding of the monoanion (Byers & Wolfenden, 1973), enzyme-bound inhibitor is dianionic, with the lost proton being taken up by the enzyme upon binding (Palmer et al., 1982).

The decreased acidity of the α-carboxylic acid of substrate VI when bound to β-lactamase I is consistent with its being proximal to an anion or to a nonpolar area in the enzyme active site. Another observation that supports the presence of an anionic residue or a nonpolar region at the active site of *B. cereus* β-lactamase I comes from the study of Kiener & Waley (1978) on reversible competitive inhibitors of this enzyme. Decarboxylation of hydrolyzed penicillins (penicilloates) to the corresponding penilloates reduces the K_i values observed at pH 6.8 between 2.5- and 7-fold. The lower affinity of the ligands containing the carboxylate could be due to electrostatic repulsion from an active site anion or to difficulty in accommodating a charge within a nonpolar milieu.

A carboxylate residue has been suggested to be at or near the active site of *B. cereus* β-lactamase I on the basis of chemical modification studies (Waley, 1975). Knowles and co-workers (Fisher et al., 1980) have pointed out that the most effective irreversible inhibitors of class A β-lactamases have acidic protons at C6 of the penam nucleus and have suggested that removal of that proton by an enzymic base may play a role in the inactivation process. Removal of tritium from 6β-bromo[6α-^{3}H]penicillinate does accompany inhibition of *B. cereus* β-lactamase I (Loosemore et al., 1980). The responsible base could be an anionic hydrogen-bond-accepting residue that the present results suggest might be present at the active site of *B. cereus* β-lactamase I.

Dependence of k_{cat}/K_m on pH. Figure 2 shows the mean values of k_{cat}/K_m for VI, plotted as a function of pH and fit

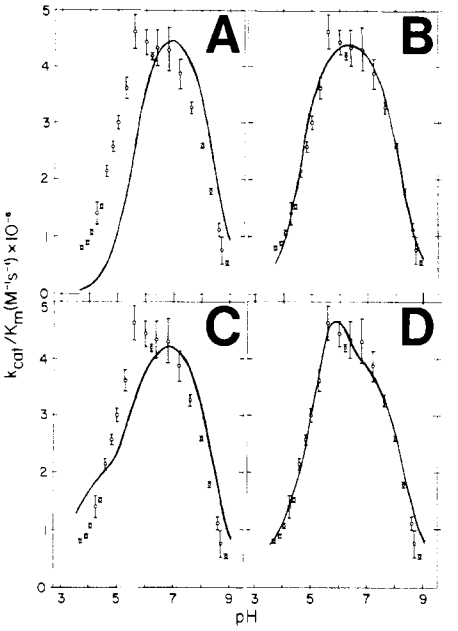


FIGURE 2: Fit of the data describing the pH dependence of k_{cat}/K_m for the *B. cereus* β-lactamase I catalyzed hydrolysis of carbenicillin (VI) to kinetic models. Experimental conditions are given in the legend to Figure 1. The points shown are the mean values (bracketed by SE's) of the data. The complete data set (65 runs) was fit by nonlinear least-squares regression to the following equations: (A) eq 2 by using fixed values of pK_{a4} = 5.55 and pK_{a5} = 8.4; the adjusted value of $(k_a)_{lim}$ so obtained is 4.8 (SE = 0.2) × 10^6 M^{-1} s^{-1}, with minimized sum of squared residuals (σ^2) = 80; (B) eq 2 by adjusting pK_{a4}, pK_{a5}, and $(k_a)_{lim}$, for which the least-squares values are respectively 4.62 (0.05), 8.08 (0.04), and 4.5 (0.1) × 10^6 M^{-1} s^{-1}, with minimized σ^2 = 8; (C) eq 3 by using fixed values of pK_{a4} = 5.55, pK_{a5} = 8.4, and pK_c = 3.19; the adjusted values of $(k_{a,\alpha\text{-COOH}})_{lim}$ and $(k_{a,\alpha\text{-COO}^-})_{lim}$ so obtained are 4.3 (0.4) × 10^8 M^{-1} s^{-1} and 4.4 (0.2) × 10^6 M^{-1} s^{-1}, respectively, with minimized σ^2 = 30; (D) eq 9 by using fixed values for pK_{a4}, pK_{a5}, and pK_c as in (C); the adjusted values of $(k_{a,\alpha\text{-COOH}})_{lim}$ and $(k_{a,\alpha\text{-COO}^-})_{lim}$ are respectively 2 (1) × 10^8 M^{-1} s^{-1} and 3.8 (0.1) × 10^6 M^{-1} s^{-1} (see Table III and the text for discussion), with minimized σ^2 = 9. The solid lines are the theoretical curves calculated from the appropriate equations by using the least-squares values of the adjustable parameters.

to several kinetic models. The theoretical curves in panels A and B of Figure 2 were obtained by nonlinear least-squares regression of the data on eq 2. The pK_a values of free enzyme

$$\frac{k_{cat}}{K_m} = \frac{(k_a)_{lim}}{1 + 10^{pK_{a4}-pH} + 10^{pH-pK_{a5}}} \quad (2)$$

determined from other substrates [5.55 and 8.4 (Hardy & Kirsch, 1984b)] were used as constants for the calculations that resulted in Figure 2A, with $(k_a)_{lim}$ as the only adjustable parameter. The data systematically deviate from this model and indicate that the two ionic forms of carbenicillin may react with the enzyme with different values of k_{cat}/K_m (see below).

The values of pK_{a4} and pK_{a5} were then additionally unrestrained (three adjustable parameters) to give the least-squares fit to the data for which the curve in Figure 2B is drawn. The pK_a values giving an optimal fit of the data to eq 2, pK_{a4} = 4.62 (0.05) and pK_{a5} = 8.08 (0.04), are substantially lower than the pK_as of free enzyme. A similar situation is found in D_2O (data not shown): the least-squares values obtained by fitting the pD profile of k_{cat}/K_m for VI to eq 2 are pK_{a4} = 5.32 (0.05) and pK_{a5} = 8.35 (0.05), whereas the pK_as of free enzyme in D_2O are 6 and 8.8 (Hardy & Kirsch, 1984b). The model of eq 2 cannot, therefore, provide a physically meaningful accounting for the pH dependence of the reaction of VI with β-lactamase I, even though the fit shown in Figure 2B is aesthetically pleasing and has a low value of σ^2.

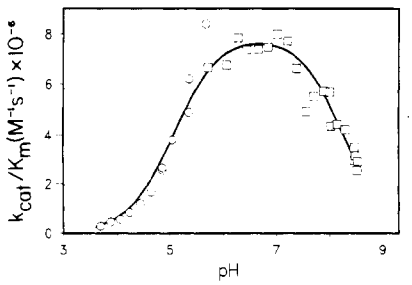
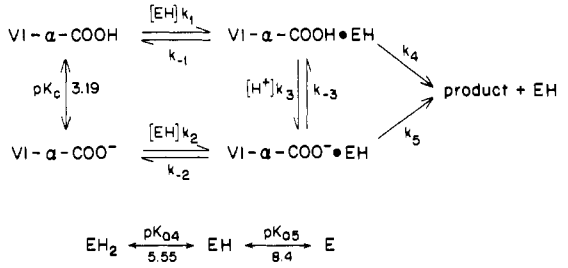


FIGURE 3: pH dependence of k_{cat}/K_m for the *B. cereus* β-lactamase I catalyzed hydrolysis of sulbenicillin (VII). Experimental conditions are given in the legend to Figure 1. Each point is the value calculated from the complete time course of one kinetic run. The data were fit by nonlinear least-squares regression to eq 2, yielding adjusted values of the parameters $pK_{a4} = 5.09$ (0.06), $pK_{a5} = 8.28$ (0.05), and $(k_a)_{lim} = 7.86$ (0.22) $\times 10^6$ M^{-1} s^{-1}. The solid line is the theoretical curve calculated from eq 2 by using the optimized paramters.

Scheme II



In contrast to these results, Waley has reported identical values for the pK_as characterizing the basic limbs of the pH vs. k_{cat}/K_m profiles for substrates VI and I [S. G. Waley, unpublished experiments reported in Abraham & Waley (1979)], the latter of which provides an intrinsic pK_a of the free enzyme (Hardy & Kirsch, 1984b).

The comparison of the k_{cat} vs. pH profiles for VI and VII discussed above helped to demonstrate that the α-COOH and α-COO^- forms of the ES complex of VI decompose to products with unequal rate constants and suggested that the analysis of the k_{cat}/K_m pH profile for VII might similarly aid in the understanding of that for VI. The pH profile for k_{cat}/K_m for VII is shown in Figure 3. The optimized pK_a values obtained from an unrestrained fit of these data to eq 2 are 5.09 (0.06) and 8.28 (0.05). These values, especially the former, are also significantly lower than the pK_as of free enzyme. However, the discrepancies are not as great for VII as are seen for VI.

The validity of eq 2 depends on the assumption that substrate ionizations in the accessible pH range play no essential role in the first irreversible step in the reaction of substrate with the enzyme (Hardy & Kirsch, 1984b). The pK_a of the α-COOH of VI is 3.19 (Zia et al., 1974). The low values of k_{cat}/K_m at the maximum of the pH profiles for both VI and VII (4.5×10^6 M^{-1} s^{-1} and 8×10^6 M^{-1} s^{-1}, respectively) compared with that for I (4.6×10^7 M^{-1} s^{-1}; Hardy & Kirsch, 1984b) suggested that both ionic forms of VI bind productively to form the ES complex, but that the predominant α-COO^- form does so with a much lower rate constant. A model based on this suggestion is shown in Scheme II.

The steps denoted by k_4 and k_5 are composite and include all reactions subsequent to Michaelis complex formation. Proton transfers to and from free enzyme and free substrate are treated as equilibria compared to the rates of association of enzyme with substrates (Cleland, 1977). No a priori assumption is made regarding the rates of proton transfer to and from the Michaelis complexes. If, however, direct interconversion of the two Michaelis complexes cannot occur (i.e., $k_3[H^+]$, $k_{-3} \ll$ rate constants for all other competing steps), then the resulting expression for the pH dependence of k_{cat}/K_m is given by eq 3 (Frieden, 1958):

$$\frac{k_{cat}}{K_m} = \frac{EH}{E_t}\left[(k_{a,\alpha\text{-COOH}})_{lim}\frac{[VI\text{-}\alpha\text{-COOH}]}{[VI]_t} + (k_{a,\alpha\text{-COO}^-})_{lim}\frac{[VI\text{-}\alpha\text{-COO}^-]}{[VI]_t}\right] \quad (3)$$

The fraction of *B. cereus* β-lactamase I that is in the active form, EH/E_t, is a function of pH and the pK_as of free enzyme (Hardy & Kirsch, 1984b):

$$\frac{EH}{E_t} = \frac{1}{1 + 10^{5.55-pH} + 10^{pH-8.4}} \quad (4)$$

[VI-α-COOH] and [VI-α-COO^-], the concentrations of VI with the α-carboxyl group protonated and unprotonated, respectively, are defined by eq 5 and 6

$$\frac{[VI\text{-}\alpha\text{-COOH}]}{[VI]_t} = \frac{1}{1 + 10^{pH-pK_c}} \quad (5)$$

$$\frac{[VI\text{-}\alpha\text{-COO}^-]}{[VI]_t} = \frac{1}{1 + 10^{pK_c-pH}} \quad (6)$$

where $[VI]_t$ is the total concentration of VI. The value of pK_c used was 3.19, the second dissociation constant reported by Zia et al. (1974). The true microscopic dissociation constant for the α-carboxylic acid of VI may be slightly different, but this does not significantly affect the calculated values of any rate constant or minimized σ^2 from the regression analyses of the pH profile of k_{cat}/K_m for VI.

The pH-independent rate constants for the first irreversible reaction of the two forms of VI with the enzyme are defined in terms of the microscopic rate constants in Scheme II by eq 7 and 8:

$$(k_{a,\alpha\text{-COOH}})_{lim} = \frac{k_1k_4}{k_{-1} + k_4} \quad (7)$$

$$(k_{a,\alpha\text{-COO}^-})_{lim} = \frac{k_2k_5}{k_{-2} + k_5} \quad (8)$$

The solid line in Figure 2C is drawn for the fit obtained by using eq 3, optimizing two parameters, $k_{a,\alpha\text{-COOH}}$ and $k_{a,\alpha\text{-COO}^-}$, the limiting rate constants for the two forms of VI combining with enzyme. The model shown in Figure 2C generates two plateau regions—the first between pH 3.2 and pH 5.5 is a result of the simultaneous loss of protons from the carboxylic acid of the substrate to give the less active form and from the enzyme to give the active form, while the second plateau between pH 5.5 and pH 8.4 is the normal one due to the ionization of the enzyme reacting with the anionic form of the substrate. As stated above, eq 3 is obtained with the restriction that interconversion of the two Michaelis complexes in Scheme II cannot occur. When this condition is relaxed, the theoretical curve obtained is the solid line shown in Figure 2D, which gives a far better fit to the data. The expression for the pH dependence of k_{cat}/K_m derived for this complete mechanism is given by eq 9

$$\frac{k_{cat}}{K_m} = \frac{EH}{E_t}(k_{a,\alpha\text{-COO}^-})_{lim}\left[\frac{1 + YH/K_c + XH^2/K_c^2}{(1 + WH/K_c)(1 + H/K_c)}\right] \quad (9)$$

where

$$Y = \frac{k_1[k_{-2}k_4(k_{-1} + k_{-3}) + k_{-1}k_5(k_{-3} + k_4)]}{k_{-1}k_2k_5(k_{-1} + k_{-3} + k_4)} \quad (9a)$$

$$X = \frac{k_1^2k_{-2}k_{-3}k_4}{k_2^2k_{-1}k_5(k_{-1} + k_{-3} + k_4)} \quad (9b)$$

$$W = \frac{k_1k_{-2}k_{-3}(k_{-1} + k_4)}{k_{-1}k_2(k_{-2} + k_5)(k_{-1} + k_{-3} + k_4)} \quad (9c)$$

The parameters Y, X, and W are lumped ratios of the microscopic rate constants shown in Scheme II. The ratio of the parameters X (eq 9B) and W (eq 9C) is equivalent (eq 10) to the ratio of the intrinsic second-order rate constants for the reaction of *B. cereus* β-lactamase I with the two forms of VI (cf. eq 7 and 8):

$$\frac{X}{W} = \frac{(k_{a,\alpha\text{-COOH}})_{lim}}{(k_{a,\alpha\text{-COO}^-})_{lim}} \quad (10)$$

The pH dependence of k_{cat}/K_m for VI was analyzed by nonlinear least-squares regression of the data on eq 9, with $k_{a,\alpha\text{-COO}^-}$, Y, X, and W as the adjustable parameters. The optimized values of the parameters so obtained for data in H_2O and in D_2O are given in Table III.

The curve described by eq 9, divided by that component that is due to the ionization of free enzyme, EH/E_t (eq 11), is shown in Figure 4. This curve gives the operational value of k_a, the second-order rate constant for reaction of VI and β-lactamase I, predicted by the full model shown in Scheme II, corrected for enzyme ionizations and has as its asymptotes $(k_{a,\alpha\text{-COOH}})_{lim}$ as $[H^+] \rightarrow \infty$ and $(k_{a,\alpha\text{-COO}^-})_{lim}$ as $[H^+] \rightarrow 0$.

$$\frac{k_{cat}}{K_m}\frac{E_t}{EH} = \left[\frac{1 + YH/K_c + XH^2/K_c^2}{(1 + WH/K_c)(1 + H/K_c)}\right](k_{a,\alpha\text{-COO}^-})_{lim} \quad (11)$$

An equation of identical form to eq 9 has been derived by Cleland (1977), who has shown that eq 9 simplifies to eq 3 if either of two conditions are met: (a) neither of the two ionization states of VI is appreciably sticky[2] or (b) both ionization states of VI have identical kinetic stickiness. A consideration of the relative values of the limiting rate constants suggests that neither of these conditions applies in the present instance as $k_{a,\alpha\text{-COOH}}$ is 2×10^8 M^{-1} s^{-1} and is likely to represent a largely diffusion controlled process, as this value is larger than the corresponding one of 4.6×10^7 M^{-1} s^{-1} for I, the reaction of which has been shown to be partially diffusion controlled (Hardy & Kirsch, 1984a). On the other hand, k_a for the α-COO^- form of VI is small enough to argue that diffusive processes are of less importance. These considerations were tested experimentally by investigating the pH dependence of the sensitivity of k_{cat}/K_m for VI to η_{rel}, with the expectation that the diffusion-controlled component would become increasingly important with decreasing pH.

The sensitivity of k_{cat}/K_m for VI to viscosity is indeed a function of pH (Table IV). Increasing the relative viscosity (η_{rel}) from 1.0 to 2.9 gave only a 10% decrease in the value of k_{cat}/K_m between pH 6 and pH 7, whereas the same increase in viscosity at pH 3.8 resulted in a 30% decrease in k_{cat}/K_m (Table IV). Such pH dependence is not observed for the viscosity variation of k_{cat}/K_m for substrates I and III, which

[2] Kinetic stickiness is defined in the previous paper (Hardy & Kirsch, 1984b).

Table III: Adjusted Parameters from Nonlinear Least-Squares Regression of the pH and pD Profiles of k_{cat}/K_m for Carbenicillin (VI) on Equation 9

	$k_{a,\alpha\text{-COO}^-}$ (M^{-1} s^{-1}) × 10^{-6} (SE)	Y × 10^{-3} (SE)	X × 10^{-4} (SE)	W × 10^{-3} (SE)	$k_{a,\alpha\text{-COOH}}$[a] (M^{-1} s^{-1}) × 10^{-8} (SE)
H_2O	3.76 (0.11)	0.75 (0.16)	1.20 (0.57)	0.21 (0.07)	2.2 (1.3)
D_2O[b]	2.79 (0.12)	3.33 (0.85)	8.7 (5.3)	1.38 (0.27)	1.8 (1.1)

[a] Calculated from the values of X, W, and $k_{a,\alpha\text{-COO}^-}$ by using eq 10. [b] The value of pK_c used to fit the D_2O data to eq 9 was 3.7 [3.2, the value of pK_c in H_2O, plus 0.5, ΔpK_a for the solvent isotope effect on the pK_a of acetic acid (Bunton & Shiner, 1961)], and the values used for the pK_as of enzyme in D_2O were 6.0 and 8.8 (Hardy & Kirsch, 1984b).

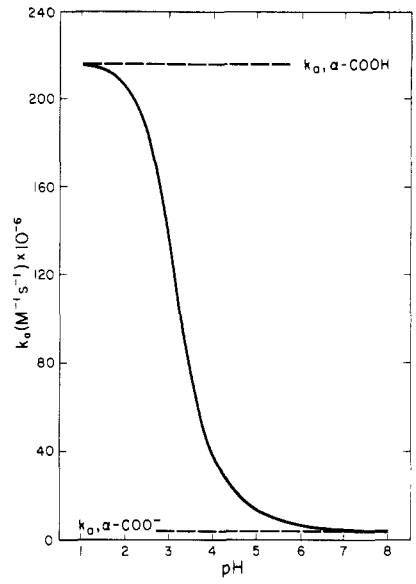


FIGURE 4: Variation with pH of the effective value of k_a for reaction of *B. cereus* β-lactamase I with carbenicillin (VI) due to different reactivities of the α-COOH and α-COO^- forms of VI, corrected for enzyme ionization. The curve was drawn from eq 11 by using $K_c = 10^{-3.19}$ and the values of the parameters listed in Table III.

apparently have only one kinetically significant ionization state (Hardy & Kirsch, 1984a). The pH dependence of the kinetic stickiness of VI cannot therefore be due to effects on the enzyme, nor can it be attributed to effects of added sucrose on substrate pK_as since the titration curves of VI obtained in 0% and ca. 28% (w/w) sucrose are nearly superimposable.

The complexity of the explicit expression for k_{cat}/K_m for VI in terms of microscopic rate constants precludes a rigorous application of the straightforward analysis leading to estimates of k_1^0 and k_{-1}^0/k_2 (Hardy & Kirsch, 1984a), which is possible with substrates having only one active form. However, at and above pH 6 most of the initial reaction of VI with β-lactamase I proceeds (see Figure 4) through the α-COO^- form of the substrate and Michaelis complex (lower pathway in Scheme II).[3] The simple analytical treatment of the viscosity dependence of k_{cat}/K_m (Hardy & Kirsch, 1984a) is adequate for the reaction of substrate VI at pH ≥6. Applying that analysis to the data listed in Table IV yields values of the partition ratio

[3] The "initial reaction" is meant to include those steps up to and including the first irreversible step of the reaction. Arguments that this step is distinct from the one that limits the rate of the overall catalyzed hydrolysis of other substrates are presented in the preceding paper (Hardy & Kirsch, 1984b).

Table IV: Effect of pH on the Sensitivity of the Kinetic Parameters for the *B. cereus* β-Lactamase I Catalyzed Hydrolysis of Carbenicillin (VI) to Relative Viscosity (η_{rel})

η_{rel} (% sucrose, w/w)	k_{cat} (s^{-1}) (SE)	K_m (M) $\times 10^4$ (SE)	k_{cat}/K_m ($M^{-1}s^{-1}$) $\times 10^{-6}$ (SE)	k_{cat}/K_m (norm)[c]
		pH 7.0[a]		
1.0	623	1.51	4.12	
(0)	(7)	(0.04)	(0.09)	0.92
2.86	712	1.88	3.78	
(28.5)	(9)	(0.02)	(0.02)	
		pH 6.5[a]		
1.0	848	1.88	4.50	
(0)	(9)	(0.02)	(0.05)	0.91
2.86	933	2.29	4.08	
(28.5)	(5)	(0.01)	(0.02)	
		pH 6.0[a]		
1.0	1310	2.97	4.42	
(0)	(20)	(0.03)	(0.04)	0.88
2.86	1340	3.44	3.90	
(28.5)	(30)	(0.09)	(0.04)	
		pH 3.8[b]		
1.0			1.41	
(0)			(0.03)	0.70
2.87			0.989	
(28.8)			(0.026)	

[a] In potassium phosphate/pyrophosphate buffers, $I_c = 0.2$, 25 °C, with [β-lactamase] = 4.18 nM and [VI] = 0.628 mM. [b] In potassium acetate/sulfate buffers, I_c = ca. 0.2, 25 °C, with [VI] = 0.142 mM. At pH 3.8, K_m for VI is greater than 2 mM; therefore, the reported values of k_{cat}/K_m were calculated from the slope of a plot of the apparent first-order rate constants vs. enzyme concentration by suing [β-lactamase] = 5.04–15.6 nM. [c] Defined as $(k_{cat}/K_m)_{\eta_{rel}} = 2.86(7)/(k_{cat}/K_m)_{\eta_{rel}} = 1.00$.

k_{-1}^0/k_2 and the association rate constant k_1^0, where the superscript denotes $\eta_{rel} = 1.0$. The figures obtained at pH 6.0, 6.5, and 7.0 are the same within one SE. The mean values of the parameters in that pH range (with the SE of the three determinations) are $k_{-1}^0/k_2 = 11.1$ (0.9) and $k_1^0 = 5.1$ (0.4) $\times 10^7$ M^{-1} s^{-1}. Thus the kinetic stickiness of VI above pH 6 is intermediate between those of substrates I and III (Hardy & Kirsch, 1984a), as might be expected since the value of $k_{a,\alpha\text{-COO}^-}$ lies between the limiting values of k_{cat}/K_m for I and III. The value of the rate constant for association of the α-COO^- form of VI with *B. cereus* β-lactamase I ($k_1^0 = 5 \times 10^7$ M^{-1} s^{-1}) is similar to that observed for other substrates (Hardy & Kirsch, 1984a). This requires that the lower value of k_a for the α-COO^- form of VI compared to that for I be due to a relative decrease in the rate of the first chemical step (k_2) or to an increase in the rate of dissociation of VI from the active site (k_{-1}). The α-COOH form of VI combines with enzyme at a rate that is more nearly diffusion controlled than is the rate for the α-COO^- form of VI. However, the interconnected parallel pathways existing at pH 3.8 preclude a meaningful quantitative analysis in terms of changes in the values of the microscopic rate constants k_1 and k_{-1}/k_2.

The model in Scheme II might also explain the perturbed pK_a values that characterize the pH dependence of k_{cat}/K_m for VII. The differences between those apparent pK_as and the true pK_as of free enzyme are less than is seen for VI, due to the much greater acidity of the α-sulfonic acid of VII compared to the α-carboxylic acid of VI; therefore, only a tiny fraction of substrate VII is ever present in the α-SO_3H form at pH values where *B. cereus* β-lactamase I is active. Even if that form of VII reacts with the enzyme with a rate constant equal to the diffusion-controlled limit, the upper pathway in Scheme II must play a much less significant role than it does for VI. Quantitative analysis of the slight perturbations is, as a result, difficult and is impeded further by the lack of a measured value for the pK_a of the α-SO_3H of VII. Possible estimates for this pK_a can be obtained from the published values for sulfoacetic acid [pK_a = 0.24 (Backer, 1923)] or methane sulfonic acid [pK_a = −0.6 (Brownstein & Stillman, 1959)]. The use of either of the values to fit the data of Figure 3 to eq 9 leads to an estimate of $k_{a,\alpha\text{-SO}_3H} \geq 10^8$ M^{-1} s^{-1} with very large standard errors because of the large extrapolation involved. Thus, the perturbation in the pK_a values characterizing the pH dependence of k_{cat}/K_m for VII from those of the free enzyme can be accounted for qualitatively at best by the contribution from the α-SO_3H form.

Table V: Solvent Deuterium Isotope Effects on the Kinetic Parameters for the *B. cereus* β-Lactamase I Catalyzed Hydrolyses of Carbenicillin (VI) and Sulbenicillin (VII)

substrate	^DV[a] (SE)	$^D(V/K)$[a] (SE)
VI (α-COOH)	1.83	1.24
	(0.06)	(1.08)
VI (α-COO^-)	1.43	1.35
	(0.21)	(0.07)
VII[b]	1.59	1.39
	(0.04)	(0.13)

[a] The values for VI are calculated from the limiting rate constants obtained from regression of the pH and pD profiles for k_{cat} on eq 1 and of k_{cat}/K_m on eq 9. [b] Calculated from four determinations each at pH 6.8 and pD 6.8, in potassium phosphate/pyrophosphate buffers, $I_c = 0.2$, with [β-lactamase] = 17.3 nM and [VII] = 249 μM.

The cases described here are, to our knowledge, the first in which it has been possible to analyze explicitly the kinetic behavior of an enzyme that discriminates nonexclusively between different protonic forms of substrates. Ionizable substrates and inhibitors have usually been found to bind to enzymes exclusively in a single protonic form, where their behavior has been examined in sufficient detail (e.g., fumarase (Massey, 1953), pepsin (Knowles et al., 1969), carboxypeptidase A (Byers & Wolfenden, 1973), dihydrofolate reductase (Feeney et al., 1975), triosephosphate isomerase (Hartman et al., 1975; Webb et al., 1977)). While the data presented in this and the previous papers (Hardy & Kirsch, 1984a,b) show conclusively that only one protonic form of the class A *B. cereus* β-lactamase I enzyme is kinetically competent, Bicknell et al. (1983) have shown that more than one ionization state of the class B and class C β-lactamases are capable of catalyzing the hydrolysis reaction.

Solvent D_2O Kinetic Isotope Effects (SKIE's). Table V contains the values of ^DV[4] and $^D(V/K)$[4] for the *B. cereus* β-lactamase I catalyzed hydrolyses of substrates VI and VII. The difference between ^DV and $^D(V/K)$ for other substrates is consistent with a two-step catalytic mechanism for *B. cereus* β-lactamase I catalyzed hydrolyses of those substrates (Hardy & Kirsch, 1984b). Such a significant difference is not evident in the calculated values of ^DV and $^D(V/K)$ for VI and VII. The imprecision in the calculated value of $^D(V/K)$ for the α-COOH form of VI prevents the interpretation of that figure. However, $^D(V/K)$ for VII and for the α-COO^- form of VI are higher than were observed for other substrates (Hardy & Kirsch, 1984b). The reason for this is unclear, although the possibility exists for a change in the nature of the transition state for the

[4] Defined in Hardy & Kirsch (1984b).

step that controls the rate of the first irreversible reaction in catalysis for these dianionic compounds. The rate of that step for VI and VII could be partially limited by proton transfer, in contrast to what one obtains for I and some other substrates (Hardy & Kirsch, 1984b). The dramatic effect of anionic substituents at the α-position of benzylpenicillins on the value of k_{cat}/K_m, which is decreased even more than is k_{cat}, is consistent with this possibility.

Added in Proof

The enzyme dihydrofolate reductase has been recently shown (Stone & Morrison, 1983) to discriminate between different protonic forms of some inhibitors such as methotrexate, binding one form preferentially but not exclusively to yield enzyme–inhibitor complexes that can interconvert by loss or gain of a proton.

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