Thermodynamic Evaluation of a Covalently Bonded Transition State Analogue Inhibitor: Inhibition of β-Lactamases by Phosphonates†

Rajesh Nagarajan and R. F. Pratt*

Department of Chemistry, Wesleyan University, Middletown, Connecticut 06459

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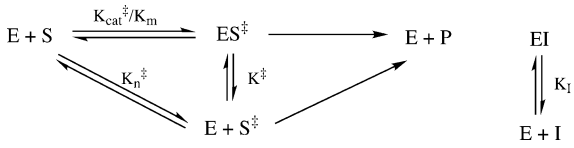
ABSTRACT: Serine β-lactamases are inhibited by phosphonate monoesters in a reaction that involves phosphonylation of the active site serine residue. This reaction is much more rapid than the hydrolysis of these inhibitors in solution under the same conditions. The β-lactamase active site therefore must have the ability to stabilize not only the anionic tetrahedral transition states of the acyl transfer reactions of substrates but also the pentacoordinated transition state(s) of phosphyl transfer reactions. A series of *p*-nitrophenyl arylphosphonates have been synthesized and the rate constants for their inhibition of the class C β-lactamase of *Enterobacter cloacae* P99 determined. There is no direct correlation between these rate constants and the dissociation constants of analogous aryl boronic acids, where the latter are believed to generate good tetrahedral transition state analogue structures. Thus, the mode of stabilization of pentacoordinated phosphorus transition states by the β-lactamase active site is qualitatively different from that of tetrahedral transition states. Molecular modeling suggests that the difference arises from different positioning of the side chain and of one of the oxygen ligands. In principle, the quality of the stable tetrahedral phosphonate complex as a transition state analogue structure can be assessed from the effect of its formation on the stability of the protein. Phosphonylation of the P99 β-lactamase, however, had little effect on the stability of the protein, as measured both by thermal and guanidine hydrochloride denaturation. Consideration of the results of similar experiments with the *Staphylococcus aureus* PC1 β-lactamase, where considerable stabilization is observed in thermal melting and, to a lesser degree, in formation of the molten globule in guanidine hydrochloride, but not in the complete unfolding transition in guanidine, suggests that results from the method may be strongly influenced by the interactions of the ligand with its environment in the unfolded state of the protein. Thus, quantitative estimates of the quality of a covalently bonded transition state analogue cannot generally be achieved by this method.

There is now a substantial history of application of the concept of the transition state analogue to enzyme inhibitor design (*1*). The general idea that, just as enzymes tightly bind specific transition states, so too will they tightly bind stable molecular analogues of these fleeting species was introduced by Pauling (*2*) and elaborated by Wolfenden (*3*). Although it might seem unlikely that a perfect transition state analogue could be synthesized as a stable molecule, it appears that a number of quite good approximations have been found (*1*).

The quality of such an inhibitor, that is, the degree to which it resembles the transition state, can be judged by a thermodynamic criterion (Scheme 1, where S and P are the substrate and product, respectively, of the enzyme E, $ES^{‡}$ is the transition state of the enzyme-catalyzed reaction, $S^{‡}$ is the transition state of the noncatalyzed reaction, and I is the potential transition state analogue inhibitor). If I were a perfect transition state analogue, then eq 1 should apply; that is, a perturbation of the system should change the free energy

Scheme 1



of transition state binding by the same amount as it does that of inhibitor binding (*4*).

$$\delta\Delta G^{‡} = \delta\Delta G_I \quad (1)$$

The free energies of eq 1 may be obtained experimentally from eqs 2 and 3; the latter assumes that, in the case under

$$\delta\Delta G^{‡} = -RT\,\delta \ln k_{cat}/K_m \quad (2)$$

$$\delta\Delta G_I = RT\,\delta \ln K_I \quad (3)$$

consideration, $\delta\Delta G_n^{‡} = -RT\,\delta \ln K_n^{‡} = 0$ (*5*). The perturbation involved could be either physical or chemical; those most used to date involve perturbation of the structure of the inhibitor (*5*) or of the enzyme (*6*).

Boronates have been found to be very effective transition state analogue inhibitors of serine proteases (*7*, *8*) and serine

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* Corresponding author. Telephone: 860-685-2629. E-mail: rpratt@wesleyan.edu. Fax: 860-685-2211.

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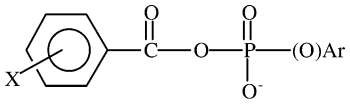
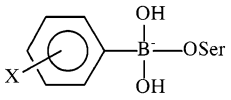
β-lactamases (*9*−*13*). Crystal structures of β-lactamases complexed with boronic acids show that the latter form tetrahedral complexes with the active site serine hydroxyl group, as would be expected of compounds yielding transition state analogue complexes (*14*−*16*). Simple aryl boronates are quite effective inhibitors of class C β-lactamases (*12*, *13*, *17*). This is somewhat surprising because aryl boronates do not closely resemble classical β-lactam substrates. Recently we showed, however, that a series of aroyl phosph(on)ates, **1**, are substrates of the class C β-lactamase



1

of *Enterobacter cloacae* P99 by virtue of favorable interaction between the phosph(on)ate leaving group and the active site (*18*, *19*). Further, aryl boronates behave thermodynamically (eq 1) as good analogues of a transition state for turnover of **1**. Since the rate-determining step for turnover of **1** is deacylation, it seems reasonable that the boronate adducts **2** are analogues of the deacylation tetrahedral intermediate **3** and associated transition states.



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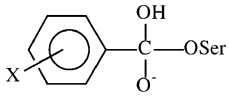

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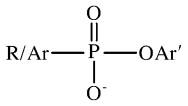
The P99 β-lactamase is also irreversibly inhibited by phosphonate monoesters **4**. These give rise to tetrahedral structures **5** (*20*), which are thought to be good transition state analogues (*21*−*23*). The phosphonate monoanions **4**



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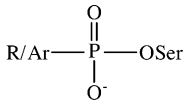

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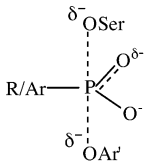
are generally quite inert to nucleophiles, much more so than comparable acyl derivatives. Their facile reaction with the class C β-lactamase active site indicates that the latter must tightly bind the presumably pentacoordinated intermediate and/or transition states **6** of the phosphonylation reaction.



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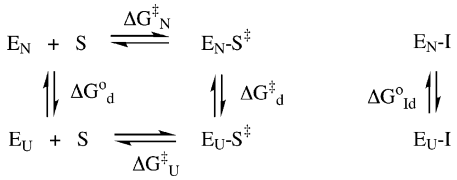
Serine proteases are also inhibited by (neutral) phosphyl species and must also tightly bind pentacoordinated transition states. The structural and functional basis for this stabilization, for either class of enzyme, is not completely understood (*20*, *24*−*26*). A relevant question, however, is whether the interactions that stabilize the tetrahedral transition states and transition state analogues are the same as those stabilizing the pentacoordinate species. Both are oxyanions which may, in principle, be electrostatically stabilized by the same active site functional groups. On the other hand, the somewhat different distribution of charges in the pentacoordinated species may lead to a differential effect. In this paper we use an extension of eq 1 to quantitatively investigate this issue.

Scheme 2



As discussed previously (*22*), the extension of eq 1 to covalently bonded transition state analogues involves determination of $\delta\Delta G_I$ from the effect of the inhibitor on protein stability [Scheme 2, where $\Delta G^{\ddagger}_N$ and $\Delta G^{\ddagger}_U$ represent the free energies of formation of the transition state from a substrate on reaction with the native enzyme (catalyzed) and unfolded enzyme (uncatalyzed), respectively, and ΔG°_d and $\Delta G^{\ddagger}_d$ represent the free energies of unfolding the free enzyme and the enzyme bound to the transition state, respectively; $\Delta G^{\ddagger}_d$ can therefore be obtained from measurements of $\Delta G^{\ddagger}_N$, $\Delta G^{\ddagger}_U$, and ΔG°_d (*22*)]. The usefulness and generality of this approach is further explored in this paper, where the quality of stable tetrahedral phosphonate complexes as transition state analogues is assessed.

MATERIALS AND METHODS

The class C β-lactamase of *Enterobacter cloacae* P99 and the class A β-lactamase of *Staphylococcus aureus* PC1 were obtained from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.) and used as received. The substrate cephalothin was a gift from Eli Lilly and Co.; benzylpenicillin was purchased from Sigma Chemical Co. Naphthalene-2-boronic acid, *m*-nitrobenzeneboronic acid, benzothiophene-2-boronic acid, and biphenyl-4-boronic acid were purchased from Lancaster, and *p*-nitrobenzeneboronic acid was purchased from CombiBlocks. Benzeneboronic acid, *p*-nitrophenyl phenylphosphonate, and the chemicals for synthesis were obtained form Aldrich Chemical Co.

m-Nitrophenylphosphonic acid was prepared by nitration of phenylphosphonic acid (*27*) and *p*-nitrophenylphosphonic acid by reaction of *p*-nitrobenzenediazonium tetrafluoroborate with PCl_3 in the presence of cuprous bromide, followed by hydrolysis (*28*). Naphthalene-2-phosphonic acid and biphenyl-4-phosphonic acid were prepared from the corresponding bromides by reaction with triethyl phosphite in the presence of nickel chloride (*29*); the resulting diethyl phosphonates were purified by distillation and hydrolyzed to the corresponding phosphonic acids in concentrated hydrochloric acid. Benzothiophene-2-phosphonic acid was prepared by reaction of benzothiophene with butyllithium at −40 °C, followed by diethylphosphoryl chloride (*30*); the resulting diester was hydrolyzed to the phosphonic acid as above. *p*-Nitrophenyl esters of the phosphonic acids, **P1**−**P6**, were prepared by direct condensation of *p*-nitrophenol with the acids in the presence of trichloroacetonitrile (*31*, *32*) and purified by recrystallization from water or by Sephadex G-10 or LH-20 chromatography. The final compounds were pure, as judged by ^{1}H and ^{31}P NMR spectra. Sodium *m*-nitrophenyl *N*-(phenylacetyl)aminomethylphosphonate, **P7**, was available from previous research in this laboratory (*32*).

Kinetics. The second-order rate constants for inactivation of the P99 β-lactamase by *p*-nitrophenyl phosphonates could be obtained either from determinations of enzyme activity as a function of time after enzyme and inhibitor were mixed or directly by spectrophotometry, by monitoring release of *p*-nitrophenoxide. The two methods gave the same values for the rate constants, within experimental error, so the direct spectrophotometric method, being more convenient, was routinely employed. Typically, enzyme from a concentrated stock solution was added to a solution of the phosphonate inhibitor in 20 mM MOPS[1] buffer, pH 7.5, 25 °C (total volume, 300 μL; final enzyme concentration, 5 μM; inhibitor concentration, 50−500 μM). The pseudo-first-order release of *p*-nitrophenoxide was monitored at 400 nm (Hewlett-Packard HP8453 spectrophotometer). Pseudo-first-order rate constants were obtained from nonlinear least-squares fitting of the data, and thence, second-order rate constants from plots of these constants versus inhibitor concentration. The latter were linear, indicating the absence of significant noncovalent binding between enzyme and inhibitors at the concentrations of the latter employed.

Aryl boronic acids are known to be competitive inhibitors of class C β-lactamases (*17*). Inhibition constants for the aryl boronic acids **B1**−**B6** and the P99 β-lactamase were determined as follows. Aliquots (5 μL) of a stock solution (1 μM; stabilized with 1% bovine serum albumin) of the P99 β-lactamase were added to cuvettes containing the substrate cephalothin (200 μM) and boronate inhibitor (0−200 μM) in 25 mM MOPS buffer, pH 7.5, 25 °C, total volume 1.0 mL. Initial rates were then determined spectrophotometrically at 278 nm. Plots of initial rates versus inhibitor concentration were fitted to a competitive inhibition equation (eq 4) by a nonlinear least-squares procedure to obtain K_i values; the K_m value of cephalothin was taken to be 20 μM.

$$v_0 = V_{max}[S]/\{K_m(1 + [I]/K_i) + [S]\} \quad (4)$$

A number of rate measurements were also carried out in a buffer consisting of 50 mM phosphate, 800 mM potassium chloride, and 45% (v/v) ethylene glycol (Aldrich, spectrophotometric grade), pH 7.5 (*33*). Steady state kinetics parameters in this solvent were obtained spectrophotometrically for cephalothin hydrolysis at both 25 and 40 °C. These parameters were then used to obtain K_I values for the boronic acids under the respective conditions.

The rate of thermal denaturation of the P99 β-lactamase was determined from fluorescence measurements from a Fluoromax-2 spectrofluorimeter. A small aliquot of the enzyme (final concentration, 0.2 μM) was added to a cuvette containing MOPS buffer that had been temperature equilibrated at 60 °C. The ensuing solution was excited at 280 nm and the fluorescence emission intensity at 350 nm monitored as a function of time. The rate constant for denaturation was then obtained by fitting these data to an equation for a single-exponential decay. This experiment was also performed with enzyme that had been inhibited by **P7**. Essentially the same procedure was employed for the PC1 β-lactamase, although different temperatures were employed (see below).

Thermodymamics. Thermal melting experiments were performed employing a Hewlett-Packard 8452A diode array spectrophotometer equipped with a temperature controller and Peltier junction temperature-controlled cuvette holder; the cuvette contained a temperature sensor and magnetic stirrer. Solutions of the P99 β-lactamase (1.4 mL, 1.0 μM) in 50 mM phosphate buffer containing 800 mM potassium chloride and 45% (v/v) ethylene glycol (*33*) were stirred (400 rpm) and heated at a rate of 0.3 °C/min, and absorption spectra (220−320 nm) were recorded at 0.3 °C intervals. The absorbances at 280 nm were plotted as a function of temperature, giving smooth, reversible melting curves where the midpoint temperatures, T_m, were independent of protein concentration (0.5−2.0 μM). T_m values and enthalpies of melting were then obtained from fitting these data to eq 5 (*34*) by means of the Kaleidograph nonlinear least-squares

$$A = [(N + yT) + (U + zT) \exp a]/[1 + \exp a] \quad (5)$$

program. In eq 5, $a = \Delta H_m[(T/T_m) - 1]/RT$ and ΔH_m is the enthalpy of melting at T_m. The other terms in eq 5 describe the linear changes of A with T before and after the melting transition. The free energy of melting at 40 °C was then calculated by means of eq 6 (*34*), where the heat capacity

$$\Delta G = \Delta H_m(1 - T/T_m) + \Delta C_p[T - T_m - T \ln(T/T_m)] \quad (6)$$

change, ΔC_p, was assumed to be constant over the temperature range under consideration. A value of 15 cal deg^{-1} per residue was assumed for ΔC_p (*35*); the P99 β-lactamase contains 360 residues, and thus, ΔC_p = 5.4 kcal deg^{-1}. Average values of T_m and ΔG from at least three separate experiments are reported.

To prepare the inactivated P99 β-lactamase, a solution of the enzyme (20 μM) was incubated with the phosphonate (50−200 μM) for at least 10 inactivation half-lives. The residual activity was assessed spectrophotometrically against cephalothin. Subsequently, the solution was dialyzed overnight against deionized water at 4 °C. The concentration and activity (essentially zero) of the enzyme were then determined spectrophotometrically and the thermodynamics of thermal melting examined as described above, where the enzyme was diluted into the MOPS buffer.

The thermal melting behavior was also studied by circular dichroism (CD); CD spectra were obtained from a Jasco J-810 spectropolarimeter. Samples of the enzyme or inactivated enzyme (1.0 μM) in the ethylene glycol-containing buffer were heated in 2.5 °C increments at a heating rate of 14 °C/h with 5 s delay times. The CD signal intensity at 220 nm was then plotted against temperature, giving smooth melting curves. These data were also fitted to eq 5. Guanidine hydrochoride (Fluka) stock solutions (7.4 M) were prepared by a literature method (*36*). The native P99 β-lactamase (5.1 μM), in 20 mM MOPS buffer at pH 7.5, was incubated for 24 h to reach equilibrium with a number of different concentrations of guanidine hydrochloride. CD spectra of the solutions were then recorded at 25 °C. Plots of the CD signal at 220 nm versus guanidine hydrochloride concentration yielded smooth denaturation curves. These data were fitted, following the method of Pace (*33*, *37*), to eq 7, yielding

[1] Abbreviations: CD, circular dichroism; MOPS, 3-morpholinopropanesulfonic acid.

$$I = (N + U \exp b)/(1 + \exp b) \quad (7)$$

$$\Delta G_d = mC_m \quad (8)$$

ΔG_d, the free energy of denaturation at zero guanidine concentration, and m, the parameter describing the linear increase of ΔG_d with guanidine concentration; the midpoint guanidine concentration of the transition, C_m, was then obtained from eq 8. In eq 7, $b = (-\Delta G_d + m[D])/RT$, where D is the denaturant, guanidine hydrochloride. These measurements were also made with enzyme inhibited by **P7**.

The denaturation of the PC1 β-lactamase in guanidine hydrochloride at 25 °C in 20 mM MOPS buffer, pH 7.5, was also studied by circular dichroism, essentially as described above. The protein concentration was 9.2 μM, and the CD signal at 220 nm was analyzed. Inactivation of the PC1 β-lactamase by **P7** (5 mM) was achieved essentially as described above for the P99 enzyme; CD measurements of its denaturation in guanidine hydrochoride were then obtained, as described.

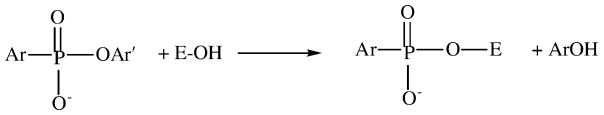
The unfolding of the PC1 β-lactamase and its complex with **P7** in guanidine hydrochloride were also studied by UV spectroscopy. The native enzyme samples, after dilution into the MOPS buffer (final enzyme concentration, 10.0 μM), were incubated with various concentrations of guanidine hydrochoride for 10 min (the first transition—see below—reached equilibrium in this time frame), and UV spectra were recorded. The difference $[(A_{276}/A_{286}) - (A_{276}/A_{286})°]$, where the latter term refers to the initial spectrum at zero guanidine concentration, was plotted as a function of guanidine hydrochloride concentration and fitted to eq 7. A similar procedure was used for the inactivated enzyme although the position of equilibrium was more slowly reached in that case and so the spectra were recorded after 24 h.

Molecular Modeling. The computations were set up essentially as previously described (*23*) and run on an SGI Octane 2 computer with Insight II 2000 (MSI, San Diego, CA). The starting point for the simulations was the crystal structure of the P99 β-lactamase with a phosphonate inhibitor covalently bound to the active serine residue [PDB entry 1BLS (*20*)]. The phosphonate ligand in this structure was replaced by **7** and **8**, representing the four- and five-coordinated phosphonate ligands, respectively. Structures and partial charges for these species were derived from MNDO calculations (MOPAC 6.0) on an appropriate liganded tripeptide (*23*). In the case of the complex of the enzyme with five-coordinated phosphorus, the positions of the phosphorus atom and the atoms bound to it were fixed, since molecular mechanics parameters for this type of structure were not available. The dianion of **8** (with neutral Tyr 150) was chosen because it represents a close analogue of the intermediate immediately formed on general base (Tyr 150 anion) catalyzed attack of the serine hydroxyl group on P1. Crystallographic waters and a 15 Å sphere of water, centered at Ser 64 O_γ, were included in the model. A short molecular dynamics run (20 ps) to relax the local structure was followed by molecular mechanics energy minimization on a typical snapshot.

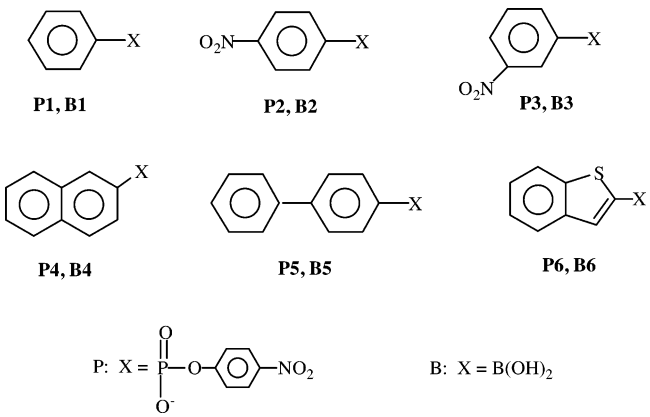
RESULTS AND DISCUSSION

The class C β-lactamase of *Enterobacter cloacae* P99 was inactivated irreversibly by the aryl phosphonate esters **P1**—**P6**. Inactivation was correlated with release of 1 equiv, with respect to enzyme, of *p*-nitrophenol. It was assumed, reasonably from all precedents (*20*, *21*), that this inactivation derives from phosphonylation of the active site serine hydroxyl group (Scheme 3). Second-order rate constants, k_i, for this inactivation reaction at 25 °C are given in Table 1.

Scheme 3



These data show that the phosphonates **P1**—**P6** rather slowly inactivate the class C *Enterobacter cloacae* P99 β-lactamase. These compounds are much less reactive (some 10^4-fold) with the enzyme than the related acyl phosphates **1**, acting as acylating agents. This is due, to some extent, to the much more favorable interaction of the phosph(on)ate leaving group of **1** with the enzyme (*19*) than the *p*-nitrophenol of **P1**—**P6**. **P1**—**P6**, however, are comparable



to **1** as phosphylating agents. Also presented in Table 1 are the thermodynamic reversible inhibition constants for the aryl boronic acids **B1**—**B6** under the same conditions. These compounds rapidly and reversibly inhibit the enzyme, forming complexes with micromolar dissociation constants. A plot of the derived free energies of activation of the phosphonates in their reaction with enzyme versus the free energies of formation of the structurally analogous boronate complexes is given in Figure 1. It is clear from Table 1 that the intrinsic electrophilicity of the phosphonyl group is not a dominant contributor to the dispersion of k_i value (see, for example, the relative k_i values for **P2** and **P3**), so a correction for this has not been made (i.e., referring back to Scheme 1 and eq 2 with S as the phosphonate, it is assumed that in the present case $\delta\Delta G_n^{\ddagger}$ is small); in any event, any such correction would be largely offset by corresponding changes in the electrophilicities of the boronic acids.

Thermodynamic Evaluation of the Pentacoordinated Intermediate/Transition State. If the phosphonates I_P and boronates I_B inactivate the enzyme according to Scheme 4,

Scheme 4

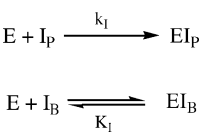


Table 1: Rate and Equilibrium Constants for Inhibition of the P99 β-Lactamase by Aryl Phosphonates and Boronates at 25 °C

phosphonate	k_i (s^{-1} M^{-1})	$\Delta G^{\ddagger}$ (kcal/mol)	boronate	K_I (μM)	ΔG_I (kcal/mol)
P1	2.90 ± 0.14	17.7 ± 0.1	**B1**	5.80 ± 1.20	7.5 ± 0.1
P2	10.8 ± 1.1	16.9 ± 0.1	**B2**	2.03 ± 0.39	8.2 ± 0.2
P3	109 ± 8	15.4 ± 0.1	**B3**	2.70 ± 0.78	8.0 ± 0.3
P4	64.9 ± 3.9	15.8 ± 0.1	**B4**	0.82 ± 0.07	8.7 ± 0.1
P5	51.1 ± 3.4	15.9 ± 0.1	**B5**	0.65 ± 0.25	8.9 ± 0.4
P6	10.3 ± 1.4	16.9 ± 0.1	**B6**	0.19 ± 0.03	9.6 ± 0.2

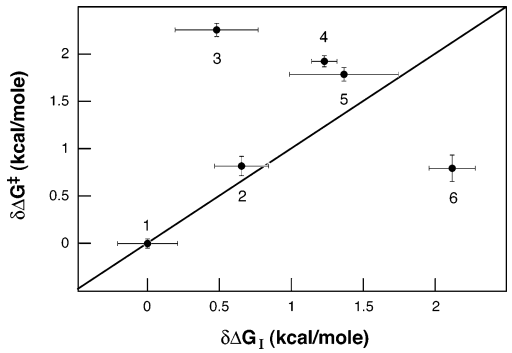


FIGURE 1: Plot of the free energy perturbation produced by aromatic substitution on the rates of inhibition of the P99 β-lactamase by *p*-nitrophenyl arylphosphonates versus that produced by the same substitutions on the dissociation constants of aryl boronates. The reference compounds are **P1** and **B1**, respectively. The diagonal line represents the fit of eq 1 to the data.

and if the transition state **6** interacts with the enzyme analogously to the tetrahedral adduct **2**, then eq 1 should hold, where the free energies of binding in this case are given by eqs 9 and 10. A plot of $\delta\Delta G^{\ddagger}$ versus $\delta\Delta G_I$ is shown in

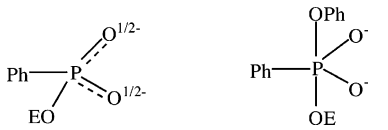
$$\delta\Delta G^{\ddagger} = -RT\,\delta \ln k_i \quad (9)$$

$$\delta\Delta G_I = RT\,\delta \ln K_I \quad (10)$$

Figure 1. Clearly, is it not well-described by a straight line of slope +1. The analogous plot for aryl boronates and aroyl phosphate substrates is linear and of unit slope (*19*).

We conclude from the results described above that the pentacoordinated phosphonyl transfer transition state **6** is, in general, stabilized by the enzyme in a somewhat different way than are tetrahedral species (boronate adducts **2** and tetrahedral transition states **3** derived from substrates). To gain some insight into this difference, structural issues were addressed. Some relevant experimental data were already available. Shoichet and co-workers have obtained crystal structures of two simple aryl boronates, the *m*-aminophenyl and 2-benzothiophene derivatives, in complexes with a class C β-lactamase (*15*, *16*). They found, as anticipated, that, in each case, the boron was covalently bonded to the active site serine O_γ. On the other hand, the two complexes differed in the orientation of the aryl group. In the former case, the aryl group occupied the area of the active site that would be occupied by the leaving group of a normal substrate, while, in the latter, it occupied the usual β-lactam side chain site. Computational analysis suggested that other aryl groups would also distribute themselves between the sites (*12*).

Informed by these results, we undertook molecular modeling of tetracoordinated and pentacoordinated phosphonate adducts. We arbitrarily chose to position the aryl group in the side chain site. Figure 2 shows energy-minimized structures **7** and **8** obtained as described in the Materials and Methods. The structures show that both the four- and five-



7 **8**

coordinated phosphorus anions, where the phosphorus is covalently bonded to Ser 64 O_γ, can interact in an apparently favorable fashion with the active site. In each case, one PO^- oxygen (PO_a^-) occupies the oxyanion hole (hydrogen-bonded to Ser 64 NH and Ser 318 NH) while the other (PO_b^-) is tightly hydrogen-bonded to Tyr 150 OH. In each case, Lys 67 NH_3^+ is hydrogen-bonded to Ser 64 O_γ and the side chain carbonyl of Asn 152. All of the above interactions are typical of phosphoryl derivatives of this enzyme (*21*, *23*). The phenoxide leaving group of the five-coordinated structure is directed out of the active site into solution and does not interact with the active site functional groups. The latter finding is in agreement with experiment (*38*). The major differences between the structures of Figure 2 lie in the positions of the PO_b^- oxygen atom and the direction of the C−P bond in the phenyl side chain. The positioning of the former, enforced by the occupation of the oxyanion hole by PO_a^-, leads to a movement of Tyr 150, and the backbone loop supporting it, further away from the B-2 strand, in effect opening up the active site. The different orientation of the phenyl ring would ensure that any substituents on it, as in **P2**−**P6**, would, in general, interact differently with the residues surrounding the active site than those on the analogous tetrahedral adduct. Cooperative interaction between these interactions and the loop bearing Tyr 150 could certainly lead to a different linear free energy relationship on equivalent perturbation of the four- and five-coordinated phosphorus adducts or, as observed experimentally in this work, between the five-coordinated phosphorus and four-coordinated boron adducts. It seems likely that if the aryl group were placed in the leaving group site rather than in the side chain site, the aryl group and one phosphoranyl oxygen would again be in different positions than they would be in a tetrahedral structure.

It is possible that the poor correlation observed in Figure 1 may arise from the tendency of the aryl groups of some boronate complexes and corresponding phosphonate transition states to occupy different sites (see above). It seems likely that the aryl groups of tetrahedral phosphonate and boronate complexes would occupy the same site because of their structural similarity, but it is possible that, in some cases, the intermediate pentacoordinated phosphonate complex would assume the alternative orientation. It is possible

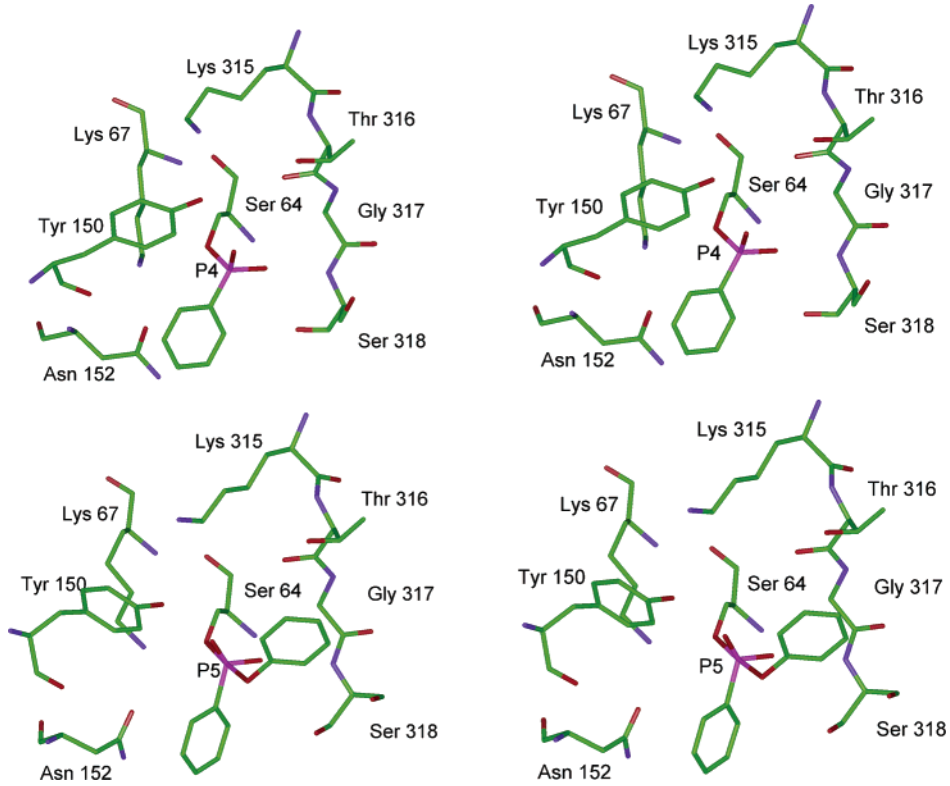


FIGURE 2: Stereoviews of energy-minimized model structures of pentacoordinated (lower pair) and tetrahedral (upper pair) phosphorus species which may occur on reaction of the P99 β-lactamase with arylphosphonates.

to draw a straight line of slope approximately +1 through four of the six points in Figure 1. This would leave the points for **P3** and **P6** as distinctive outliers. Even taking into account the issues of ligand orientation discussed above, it is difficult to achieve the dispersion obtained without a number of ad hoc assumptions. The simpler interpretation, taken above, that the penta- and tetracoordinated species interact differently with the active site, seems at least as likely.

Thermodynamic Evaluation of the Stable Tetracoordinated Phosphonyl Complex. To evaluate the tetracoordinated phosphonyl derivative **5** of the P99 β-lactamase as a tetrahedral transition state analogue structure, $\delta\Delta G_I$ of eq1 must be obtained from the effect of the phosphonylation on enzyme stability (*22*). In principle, $\delta\Delta G_I$ values determined in this way should linearly correlate with $\delta\Delta G^{\ddagger}$ values from an analogous series of substrates or, alternatively, with $\delta\Delta G_I$ values from a series of rapidly reversible transition state analogue inhibitors. Previously, in a study involving the class A *Staphylococcus aureus* PC1 β-lactamase (*22*), we used the former correlation, but the latter was more convenient in the present case; the latter does require, however, a very well-defined series of good transition state analogue inhibitors as a reference set. In the present study, we employed the aryl boronates for this purpose, since their complexes, **2**, appear to be good analogues of the transition state for the hydrolysis of the aroyl derivatives of β-lactamases derived from the aroyl phosphates (*19*).

Table 2, therefore, shows the results of experiments with the P99 β-lactamase to obtain data for the correlation described above. The thermal melting experiments, to determine the effect of phosphonylation on protein stability, were carried out in an aqueous/organic medium containing 50 mM phosphate, 800 mM potassium chloride, and 45% ethylene glycol because, as Shoichet and co-workers have found (*33*), class C β-lactamases appear to melt reversibly in this medium; in purely aqueous media, the melting is clearly irreversible. Typical melting curves from these experiments are shown in Figure 3. The boronate K_I values were determined in the aqueous ethylene glycol medium at 40 °C, a temperature close to the melting point, so that the extrapolation of thermodynamic parameters with temperature would be minimal.

Table 2: Thermodynamic Data for Inhibition of the P99 β-Lactamase by Aryl Phosphonates and Boronates at 40 °C

phosphonate	T_m (°C)	ΔG_{IP} (kcal/mol)	boronate	K_I (μM)	ΔG_{IB} (kcal/mol)
P1	52.1 ± 0.1	8.5 ± 0.3	**B1**	54 ± 12	6.1 ± 0.1
P2	52.0 ± 0.3	8.5 ± 0.6	**B2**	37 ± 13	6.3 ± 0.2
P3	52.2 ± 0.4	9.3 ± 0.2	**B3**	68 ± 18	6.0 ± 0.2
P4	53.5 ± 0.5	9.9 ± 0.2	**B4**	7.4 ± 0.5	7.3 ± 0.1
P5	52.0 ± 0.1	8.5 ± 0.3	**B5**	12.7 ± 1.4	7.0 ± 0.1
P6	52.3 ± 0.1	7.2 ± 0.1	**B6**	3.2 ± 0.2	7.9 ± 0.1
P7	53.0 ± 0.1	9.7 ± 0.2			
native	50.2 ± 0.3	7.9 ± 0.2			

The data of Table 2 show that the boronates should stabilize the enzyme, via Scheme 5, by 6−8 kcal/mol at 40 °C, depending on the substrate. This seems a reasonable value for a transition state analogue of a nonoptimal substrate. The phosphonate data, however, are more problematic. First, the data show that the aryl phosphonate adducts have melting points very close to that of the native enzyme. Second, when free energies of melting at 40 °C were calculated (Table 2), they also showed only small differences from that of the native enzyme—the phosphonates appear to stabilize the enzyme by <3 kcal/mol, and in one case, **P6**, the adduct is less stable than the native enzyme. These results suggest that

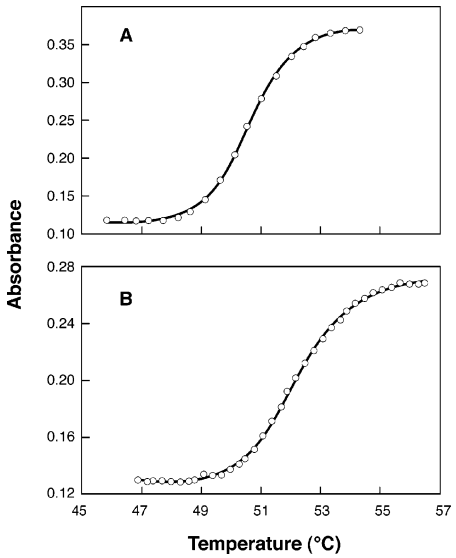
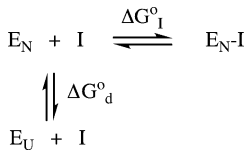


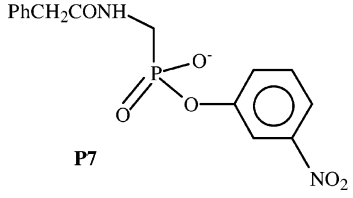
FIGURE 3: Thermal melting curves, monitored spectrophotometrically at 280 nm, for the P99 β-lactamase (A) and its **P7** derivative (B). The points are experimental data, and the lines are derived from fitting the data to eq 5. Other examples are shown as Supporting Information.

Scheme 5



the interpretation of this protein stability data is not as straightforward as previously thought (*22*).

The phosphonate data of Table 2 are very strikingly different from previous results with phosphonate adducts of another β-lactamase (*22*). Thermal melting of the complex between the class A PC1 β-lactamase and **P7** showed that the phosphonate increased the melting temperature by 13 °C with a concomitant stabilization of the enzyme of some 15 kcal/mol. Although 15 kcal/mol is certainly suggestive of the affinity of an enzyme for a transition state of an effectively catalyzed reaction, 0.75 kcal/mol (average of data for **P1**−**P6**) certainly is not. One difference between the two studies, although not one that was anticipated to be significant, is that the greatest effects in the previous study involved phosphonates such as **P7** that contain β-lactamase-specific amido side chains. To assess any effect of this difference, the covalent adduct of the P99 β-lactamase and **P7** was prepared. This phosphonate inactivates the P99 β-lactamase much more rapidly than the PC1 enzyme and thus might be expected to interact with the active site more strongly. The



P7

P7 derivative was melted under the same protocol as described above, yielding a T_m value of 53.0 ± 0.1 °C (ΔT_m = 2.8 ± 0.3 °C) and a free energy of melting of 9.7 ± 0.16 kcal/mol at 40 °C. Surprisingly, therefore, the phosphonate

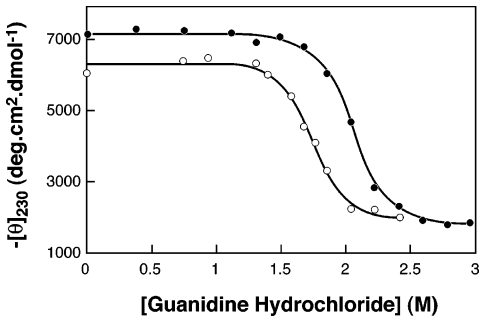


FIGURE 4: Denaturation of the P99 β-lactamase (open circles) and its **P7** derivative (closed circles) monitored by circular dichroism at 220 nm. The points are experimental data, and the lines are derived from fitting the data to eq 7.

P7, which is thought to produce a close analogue of the transition state for hydrolysis of some good substrates (*20*−*23*), does not appear to affect the stability of the β-lactamase any more than do the less specific **P1**−**P6** (Table 2). A similar phenomenon has been noticed by Shoichet et al. (*39*) with the TEM β-lactamase, a class A enzyme homologous to PC1.

Further evidence of the problem is observed in Figure 4, where guanidine hydrochloride denaturation, monitored by circular dichroism, of the P99 β-lactamase and its **P7** derivative is shown. The result is similar to that from the thermal denaturation experiments: the (specific) phosphonate **P7** had only a small effect on the stability of the enzyme—C_m (midpoint concentration) values were 1.7 ± 0.3 M and 2.0 ± 0.2 M, respectively, for the native enzyme and its phosphonate derivative, and the respective free energies of denaturation at zero guanidine concentration were 8.0 ± 1.0 kcal/mol and 8.7 ± 0.6 kcal/mol. It is noticeable, however, that the *m* values derived from these experiments (eq 8), 4.7 kcal/(mol M) and 4.35 kcal/(mol M) for the native enzyme and its **P7** derivative, respectively, are rather low for a protein of the size of the P99 β-lactamase (*33*, *40*). Given that the system was at equilibrium at all guanidine concentrations, as the spectral evidence suggested, a possible explanation of the low *m* values would be that, under the conditions employed, the unfolding process in guanidine hydrochoride actually consists of two steps with some significant accumulation of an intermediate (*41*). Even if this were true, however, the results indicate that the phosphonate does not markedly stabilize either the initial state or the intermediate. Thus, these results seem to imply that, if the phosphonates are indeed good transition state analogues, the differential interaction energies between the ligand and its environment, where the latter is initially the enzyme, are conserved in the end states of both thermal and guanidine hydrochloride-induced denaturation.

In view of these results, we returned to the PC1 β-lactamase. The **P7** derivative of this enzyme was prepared, and both it and the native enzyme were denatured in guanidine hydrochloride. The results of this experiment are shown in Figure 5. When the denaturation was monitored by far UV circular dichroism (loss of secondary structure), panel A, the results were the same as those described above for the P99 β-lactamase; viz., the phosphonate has little apparent effect on enzyme stability (C_m values were 1.6 ± 0.2 M and 1.8 ± 0.3 M, respectively, for the native enzyme and phosphonate derivative). On the other hand, panel B of Figure 5 shows

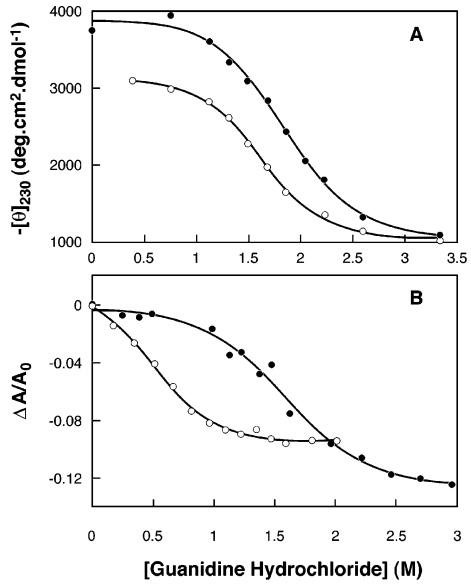


FIGURE 5: Denaturation of the PC1 β-lactamase (open circles) and its **P7** derivative (closed circles), monitored by circular dichroism at 220 nm (A) or by UV absorption at 276 nm (B). The points are experimental data, and the lines are derived from fitting the data to eq 7.

Scheme 6

$$E_N \rightleftharpoons E_H \rightleftharpoons E_U$$

that if unfolding is monitored by the UV absorption of aromatic residues (286 nm), a distinct stabilization of the protein by the phosphonate is demonstrated. Values of C_m and the free energy of the transition for the native enzyme were 0.5 ± 0.1 M and 1.3 ± 0.2 kcal/mol, respectively, and those for the phosphonate were 1.6 ± 0.2 M and 2.8 ± 0.4 kcal/mol, respectively. This stabilization is, however, much less than that observed in the thermal melting experiment (*22*).

Pain and co-workers have shown that the PC1 β-lactamase unfolds in guanidine hydrochloride in two clearly defined steps (Scheme 6). The first, which can be monitored by UV spectroscopy, as employed above, involves expansion of the protein and exposure of the aromatic residues to solvent; a molten globule E_H is formed. Loss of enzyme activity is correlated with this transition. At higher guanidine concentrations, secondary structure is lost, as revealed by the far UV circular dichroism changes (*42*−*44*).

The new results with the PC1 β-lactamase suggest a solution to the problem encountered above, viz., that of the apparently unpredictable magnitude of enzyme stabilization by the phosphonates.

In principle, the method of assessing the effect of putative transition state analogues on protein stability described above would be rigorous if the enzyme−analogue complex reversibly unfolded to a product where the analogue did not interact with its environment in a ligand-specific fashion. In reality, this situation is not strictly possible, although an approximation to it might occur where structure-specific contributions were only a small part of the total energy of interaction of the ligand with the environment in the unfolded state. Such an approximation must occur in the final state achieved on thermal melting of the phosphonate complex of the PC1 enzyme (*22*). Presumably, the specific interactions between the enzyme active site and the phosphonate are lost on thermal unfolding [although thermally "unfolded" proteins are known to often retain considerable structure (*45*, *46*)]. If the interactions between the phosphonate and its largely aqueous environment after unfolding were not strongly structure specific—differences could also be dampened by compensation phenomena [e.g. enthalpy/entropy compensation (*47*−*50*)]—then the observed phosphonate structure-specific stabilization of the protein would be achieved. In the molten globule where all of the structure-specific interactions between ligand and protein may not be lost, the apparent stabilization of the protein by the ligand may be smaller, as observed. The binding of specific ligands to molten globules is not unknown (*51*).

Also related to these results is the observation that the **P7** phosphonate adduct of the PC1 β-lactamase is kinetically considerably more stable to thermal denaturation than the apoenzyme. At 57 °C in MOPS buffer, the rate constant for denaturation of the phosphonate derivative was 1.43×10^{-3} s^{-1}. Under the same conditions, the apoenzyme denatured too rapidly to be observed by manual mixing methods (rate constant > 0.1 s^{-1}); at 45 °C, the rate constant for denaturation of the apoenzyme was 1.6×10^{-2} s^{-1}. Specific ligand−enzyme interactions must be lost, to some degree, at least, in the transition state for denaturation. It is possible, therefore, that this transition state resembles the final state in thermal unfolding; that is, it is a late transition state. In contrast, the rate constants of melting of the P99 β-lactamase and its **P7** derivative at 60 °C were 5.7×10^{-4} s^{-1} and 5.0×10^{-4} s^{-1}, respectively. The ligand-specific interactions must be conserved in the thermal denaturation transition state for this enzyme, just as they were in the final state. These results from kinetics measurements therefore appear to correlate with the thermodynamic observations made with the respective enzymes.

The situation for the PC1 enzyme at high guanidine hydrochloride concentrations must be different from that after thermal unfolding. One must assume that, for both the P99 and PC1 β-lactamases, strong ligand-specific interactions with the environment, still including the protein perhaps, are retained after "complete" unfolding in guanidine hydrochoride. A solubility experiment did, in fact, demonstrate significant interaction between a phosphonate monoanion and the mixed aqueous environments employed in the experiments described above. In this experiment, 50 mg of 2-naphthalene phosphonic acid was suspended in each of 1 mL of MOPs buffer, pH 7.5, 1 mL of MOPS buffer also containing 2.5 M guanidine hydrochloride, and 1 mL of 50 mM phosphate buffer, pH 7.0, containing 800 mM KCl and 45% ethylene glycol. The samples were incubated for 24 h, with occasional vortex mixing, and absorption spectra of the supernatant were then taken. The concentrations of the phosphonic acid in the respective solutions were 15.7, 0.5, and 2.4 mM. Hence, there were, in fact, appreciable interactions between an aryl phosphonate monoanion and guanidine hydrochloride, on one hand, and the ethylene glycol solvent, on the other. Selectivity in these interactions between different aryl groups could, partly at least, offset the stabilization differences afforded by the native enzyme active site.

Finally, there remains the case of the thermal unfolding of the P99 β-lactamase where the phosphonate has little

effect. In this instance, also, one must suppose that the structure-specific interactions between the phosphonate and its environment remain largely intact in the reversible end product state achieved. Since this result was also obtained when the transition was monitored by far UV circular dichroism, it seems likely that most of the original protein structure, including secondary structure, must be lost in the final states. Thus, specific interactions must remain between the ligand and either the unfolded protein or the solvent (aqueous ethylene glycol). The contrast between these results and those of boronic acid dissociation from the protein in the same solvent, where ligand-specific interactions must be largely lost, is striking. The difference may result from the presence of a negative charge on the phosphonate but not on the neutral boronic acid.

There are certainly a considerable number of examples of protein stabilization by covalently bonded ligands, but there are few known to us where the degree of stabilization can be quantitatively predicted and therefore where the results can be tested against expectations. Such calibrated examples as we are aware of are transition state analogues where the expected stabilization can be estimated, as described above, from substrate turnover kinetics. One example where a significant amount of transition state binding is apparently visible is the *p*-nitrophenylphosphoryl derivative of chymotrypsin (*22*). Another example is that of dehydroquinase, where a reaction intermediate/transition state analogue was attached by borohydride reduction. This modification markedly stabilized the enzyme to heat, guanidine hydrochloride, and proteolysis (*52*). Enzyme−substrate intermediates, of β-lactamases, for example, appear to be either stabilized or destabilized with respect to the apoenzyme (*33*, *39*); one would expect stabilization by good (specific) substrates.

CONCLUSIONS

The class C β-lactamase active site interacts in a different fashion with four- and five-coordinated ligands. Both are stabilized significantly but by quantitatively and qualitatively different interactions. There is therefore more than one way to occupy this active site with anionic ligands and produce effective inhibition. This conclusion is in accord with the experimental finding that five- or six-coordinated vanadium complexes are submicromolar inhibitors of the P99 β-lactamase (*53*, *54*). This information may inform further inhibitor design.

In general, it seems at present that the method of thermodynamic evaluation of covalently bonded transition state analogues by means of eq 1 and protein stability measurements is not quantitatively rigorous, since the nature and extent of interaction of the analogue with the environment after denaturation cannot readily be estimated. The more commonly employed case of noncovalently interacting transition state analogues seems better behaved (*1*, *5*), probably, to some degree at least, because the final environment is the same (purely aqueous) in both the substrate ($\delta\Delta G^{\ddagger}$) and inhibitor ($\delta\Delta G_I$) measurements. The caveats produced by these results presumably apply generally to the thermodynamic evaluation of covalent adducts of proteins by denaturation studies.

SUPPORTING INFORMATION AVAILABLE

Examples of thermal melting curves for the P99 β-lactamase and a variety of phosphonate (**P1**−**P7**) derivatives. A table of the parameters derived from fitting these data to eq 5. This material is available free of charge via the Internet at http://pubs.acs.org.

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