Characterization of Covalently Bound Enzyme Inhibitors as Transition-State Analogs by Protein Stability Measurements: Phosphonate Monoester Inhibitors of a β-Lactamase†

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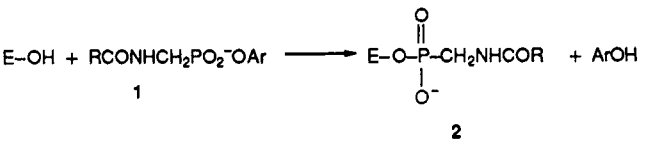
ABSTRACT: An experimental method is described for determining whether a covalent enzyme–inhibitor complex has the properties expected of a transition-state analog. The method involves a comparison of the noncovalent interaction energies between the enzyme and the inhibitor on one hand (determined from protein denaturation thermodynamics) and the analogous transition state on the other (determined from kinetic measurements). These two quantities should presumably be large (in comparison with the interaction energies of substrates or reaction intermediates) and close to equal for a good transition state analog; the former is seen dramatically in a large increase in protein stability. The method is absolute in the sense that it does not require a crystal structure of the inhibited enzyme or any preconceptions as to the mechanism of action of the enzyme except those which led to adoption of the potential transition state analog and which might turn out to be right or wrong. In this paper the method is quantitatively applied to the inhibition of the *Staphylococcus aureus* PC1 β-lactamase by phosphonate monoesters. It is concluded that the enzyme–inhibitor complex in this case is likely to be a good transition-state mimic. Therefore, mechanistic interpretation of the crystal structure of the complex can be made with more confidence. A semiquantitative assessment of the situation with serine proteinases is also made. It is concluded, in agreement with predictions based on the generally accepted mechanism and on crystal structures, that anionic, but not neutral, phosph(or/on)yl derivatives are good transition-state analogs.

Phosphonate monoesters of structure **1** inhibit β-lactamases by phosphonylation of the primary nucleophile of the β-lactamase active site, a specific serine hydroxyl group (Scheme I; Pratt, 1989; Rahil & Pratt, 1992). The kinetically inert **2** has a structure rather closely resembling that of the tetrahedral intermediate **3** arising during enzyme-catalyzed hydrolysis of the acyl-enzyme **4** generated during turnover of the substrate **5**, the carboxylic acid analog of **1**. Serine proteinases are inhibited in a similar way by phosph(or/on)yl analogs of their substrates (Aldridge & Reiner, 1972). Such inhibitors are often referred to as transition-state analog inhibitors because of the structural resemblance between **2** and **3** (Bernard & Orgel, 1959; Kraut, 1977; Ashani & Green, 1982). The phosphonates **1** were of course designed to be β-lactamase inhibitors of this type (Pratt, 1989).

If **2** were in fact a good structural analog of the tetrahedral intermediate and hence of the associated transition states of normal catalysis, one would expect to find the catalytic functional groups of the β-lactamase active site clustered about the phosphonate moiety as they would be about **3**. Indeed, crystal structures of analogous complexes of serine proteinases show just such clustering—an O^- fixed in the oxyanion hole and the general base catalyst of the active site, a histidine imidazole group, in protonated form, at the other oxygen (Stroud et al., 1974; Kossiakoff & Spencer, 1981; Harel et al., 1991); thus **6** as a mimic of **7**. Since our understanding of the mechanism of action of β-lactamases, particularly with respect to a general base catalyst, is at present in a highly speculative state (Herzberg & Moult, 1991; Lamotte-Brasseur et al., 1991; Waley, 1992; Strynadka et al., 1992; Fink, 1992), the crystal structure of an example of **2** would seem a likely source of progress. Such a crystal structure has been obtained of the inert complex formed on reaction of the class A β-lactamase of *Staphylococcus aureus* PC1 with a phosphonate monoester (**15**, see below) (Chen et al., 1993). The structure of this derivative did not, however, completely conform to the expectation of **6** [see Chen et al. (1993) and further discussion in this paper]. This result raised the question of whether the phosphonyl-enzyme **2** was indeed a transition-state analog or merely a chemically modified enzyme; any covalent modification of the active-site serine would of course inhibit the β-lactamase. The same question may also be asked of the analogous derivatives of serine proteinases, although in that case the structures of anionic phosphates certainly met all mechanistic expectations.

Scheme I



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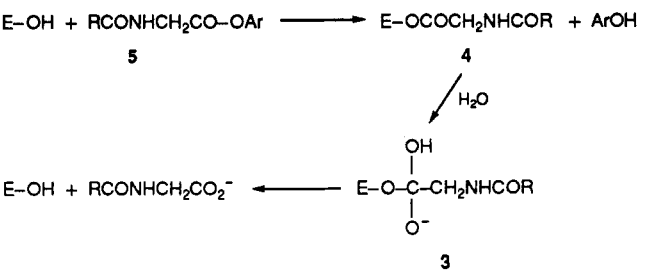
Scheme II



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A general method of demonstrating that an inhibitor is in fact a transition-state analog follows directly from the

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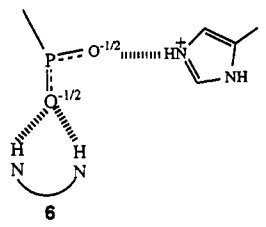

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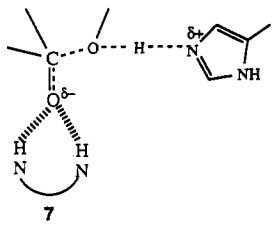

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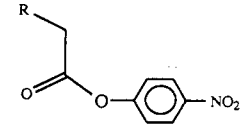
definition of such inhibitors (Wolfenden, 1972; Lienhard, 1973). If a transition state and its analog are completely isostructural, the free energy of noncovalent interactions with the enzyme should be the same in each case. A less than ideal analog, such as are often produced because of synthetic limitations, would interact less effectively with the enzyme. Further, the free energy change brought about to the complex of an enzyme and a transition-state analog by a perturbation of the system should equal that to the transition state of the enzyme-catalyzed reaction by the same perturbation. The free energy change in the latter case can, in principle, be determined from the change in an appropriate rate constant and in the former from the change in stability of the complex. This general approach has been elegantly validated by Bartlett and co-workers for reversible, noncovalent inhibitors of carboxypeptidase A, where the perturbations employed have been structural changes in either the substrate/inhibitor (Bartlett & Marlow, 1983; Hanson et al., 1989) or in the protein (Phillips et al., 1992). In each case, linear plots of unit slope were obtained between ln K_i for the inhibitor(s) *vs.* $-\ln (k_{cat}/K_M)$ for the substrate(s).

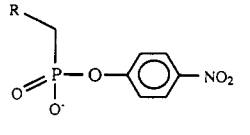
To our knowledge, however, this approach has not yet been applied to covalently attached transition-state analogs. Since we had a potential example of this type in the β-lactamase–phosphonate system, and moreover, one where the answer as to whether the inhibitor was a transition-state analog or not had intrinsic interest, as described above, we decided to investigate the applicability of these thermodynamic comparisons.

The substrates **8–11** of the *S. aureus* PC1 β-lactamase and the corresponding inhibitors **12–15** were therefore assembled. As described below, k_{cat}/K_m values for **8–11** were measured to obtain transition-state free energies. In contrast to the situation with reversible inhibition, however, the extent of noncovalent stabilization of the enzyme–inhibitor complexes was not, and should not be, determined from direct measurements of inhibitor effectiveness but from measurements of the stability of the enzyme–inhibitor complex to denaturation. The rationale for this procedure and the assumptions involved are presented below, along with the results obtained in this particular case of β-lactamase inhibition. A preliminary assessment of the procedure with the serine proteinase α-chymotrypsin was also made.

We conclude that the structures **2** most likely *do* represent transition-state analogs for β-lactamase catalysis. The implications of this finding to the β-lactamase mechanism are briefly discussed.



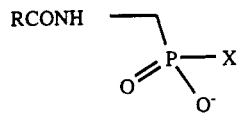
8 R = H
9 R = $NHCO_2Me$
10 R = $NHSO_2CH_2Ph$
11 R = $NHCO_2CH_2Ph$



12 R = H
13 R = $NHCO_2Me$
14 R = $NHSO_2CH_2Ph$
15 R = $NHCO_2CH_2Ph$

EXPERIMENTAL PROCEDURES

Materials. The *S. aureus* PC1 β-lactamase was obtained from the Centre for Applied Microbiology and Research (Porton Down, Wiltshire, U.K.). Bovine pancreatic α-chymotrypsin was purchased from Sigma Chemical Co. *N*-(Benzyloxycarbonyl)glycine *p*-nitrophenyl ester, tris(*p*-nitrophenyl) phosphate, *p*-nitrophenyl acetate, and *N*-benzoyl-L-tyrosine ethyl ester were purchased from Sigma Chemical Co. Aminomethanephosphonic acid and phenylmethanesulfonyl chloride were obtained from Lancaster Synthesis and methylphosphonic difluoride from Johnson Matthey Electronics. Benzyl chloroformate and methyl chloroformate were purchased from Aldrich Chemical Co. Moxalactam and 6-β-bromopenicillanic acid were gifts from Eli Lilly and Co. and Leo Pharmaceutical Products, respectively. The synthesis and purification of *p*-nitrophenyl [[*N*-(benzyloxycarbonyl)-amino]methyl]phosphonate (**15**), *p*-nitrophenyl [[*N*-(phenylacetyl)amino]methyl]phosphonate (**16**), [[*N*-(benzyloxycarbonyl)amino]methyl]phosphonyl fluoride (**17**), and *p*-nitrophenyl methylphosphonate (**12**) have been described elsewhere (Rahil & Pratt, 1991a, 1992, 1993).



16 R = $PhCH_2$, X = p-$OC_6H_4NO_2$
17 R = $PhCH_2O$, X = F

Sodium p-Nitrophenyl [[N-[(Phenylmethyl)sulfonyl]amino]methyl]phosphonate (**14**). This compound was synthesized by the coupling of the phosphonyl dichloride to *p*-nitrophenol in a fashion similar to that adopted for some of the other phosphonyl esters mentioned above. The details of this synthesis will be published elsewhere. ^{1}H NMR (D_2O): δ 3.40 (d, J = 12 Hz, 2 H, CH_2P), 4.55 (s, 2 H, CH_2SO_2), 7.42 (d, J = 10 Hz, 2 H, ArH), 8.25 (d, J = 10 Hz, 2 H, ArH), 7.47 (m, 5 H, ArH).

Sodium p-Nitrophenyl [[N-(Methoxycarbonyl)amino]methyl]phosphonate (**13**). The parent phosphonic acid was prepared by the reaction of methyl chloroformate and aminomethanephosphonic acid, as described earlier for the benzyl analog (Rahil & Pratt, 1991). The acid was coupled to *p*-nitrophenol in the presence of trichloroacetonitrile and the product purified by Sephadex G-10 gel-filtration chromatography, both as previously described (Rahil & Pratt, 1993). The product exhibited the following ^{1}H NMR spectrum: (^2H_2O) δ 3.36 (d, J = 12 Hz, 2 H, CH_2P), 3.65 (s, 3 H, CH_3O), 7.35 (d, J = 10 Hz, 2 H, ArH), 8.30 (d, J = 10 Hz, 2 H, ArH).

p-Nitrophenyl [N-[(Phenylmethyl)sulfonyl]amino]acetate (**10**). N-[(Phenylmethyl)sulfonyl]glycine was prepared from glycine and phenylmethanesulfonyl chloride and then condensed with *p*-nitrophenol in the presence of 1-[(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride, both steps by general literature procedures (Bodanszky & Bodanszky, 1984). *p*-Nitrophenol was removed from the crude product by vacuum sublimation at 80 °C and the resulting solid was recrystallized from benzene/cyclohexane (70/30). The purified product, mp 138–140 °C, yielded the following ^{1}H NMR spectrum: (C^2HCl_3) δ 4.00 (d, J = 10 Hz, 2 H, CH_2CO), 4.30 (s, 2 H, CH_2SO_2), 4.80 (br t, 1 H, NH), 7.33 (d, J = 10 Hz, 2 H, ArH), 7.4 (m, 5 H, ArH), 8.30 (d, 10 Hz, 2 H, ArH).

p-Nitrophenyl [N-(Methoxycarbonyl)amino]acetate (**9**). This compound was prepared in essentially the same way as **10**, except that the condensation was carried out in the presence

of carbonyldiimidazole (Govardhan & Pratt, 1987). The product was recrystallized from ethyl acetate/hexane and had mp 76–78 °C and the following ^{1}H NMR spectrum: (C^2-HCl_3) δ 3.85 (s, 3 H, CH_3O), 4.38 (d, J = 5 Hz, 2 H, CH_2-CO), 5.35 (br t, 1 H, NH), 7.45 (d, J = 10 Hz, 2 H, ArH), 8.40 (d, J = 10 Hz, 2 H, ArH).

Analytical and Kinetic Methods. Absorption spectra and spectrophotometric rates were obtained from a Perkin-Elmer Lambda 4B spectrophotomer. β-Lactamase activity was routinely determined against benzylpenicillin by the spectrophotometric method of Waley (1974). Concentrations of *S. aureus* PC1 β-lactamase were determined spectrophotometrically by employing a published extinction coefficient of 1.95×10^4 M^{-1} cm^{-1} at 276.5 nm (Carrey & Pain, 1978). All kinetic measurements were performed at 25 °C in 20 mM MOPS buffer at pH 7.50.

Values of k_{cat}/K_M for the substrates **8–11** were obtained from the pseudo-first-order rate constants, k_{obs}, for hydrolyses of these substrates at initial concentrations of between 0.2 and 0.5 μM in the presence of enzyme (0.1–0.2 μM). These substrate concentrations were shown to be at least 10 times smaller than K_M in each case; for example, K_M for **11**, the best of the substrates, was found to be 4.30 ± 0.03 μM. In each case it was necessary to correct for background substrate hydrolysis which was obtained as the pseudo-first-order rate constant, k_0, for hydrolysis of the substrate in the presence of the appropriate concentration of enzyme that had been completely inactivated by 6-β-bromopenicillanic acid (Pratt & Loosemore, 1978). The need to include the contribution from nonspecific, i.e., proceeding other than at the active site, enzyme-catalyzed hydrolysis was particularly important for the poorest and least specific substrate **8**. Equation 1 was used to obtain values of k_{cat}/K_M for each substrate. Solutions

$$(k_{cat}/K_M) = (k_{obs} - k_0)/E_0 \quad (1)$$

of the esters were prepared by sonication of the solid in buffer followed by removal of the undissolved material by filtration. Concentrations were estimated from absorbance measurements at 400 nm after complete hydrolysis of the esters on addition of alkali. Reactions were followed spectrophotometrically at 317 nm.

The rate constant for the uncatalyzed (or water-catalyzed) hydrolysis of *p*-nitrophenyl acetate was determined spectrophotometrically, as above, from measurements of initial rates in dilute hydrochloric acid. Rate constants in 0.01 and 0.02 M HCl were identical and therefore assumed to correspond to the uncatalyzed reaction; the value obtained, 2.0×10^{-8} s^{-1}, was similar to those from previous determinations described in the literature (Jencks & Carriuolo, 1960).

Taft Polar Substituent Constants. The σ^* values for the substituted acetyl substituents in **8–11** were obtained from experimentally determined pK_as of the corresponding substituted acetic acids. Stock solutions of these acids of about 0.02 M were prepared. Aliquots of 50 mL were titrated with standard sodium hydroxide (0.1 M) solution and pH readings were obtained from a Radiometer PHM62 pH meter equipped wtih an Ingold combination electrode. The titration profiles were fitted by means of a nonlinear least-squares program to

$$V_{base} = \frac{[(K_W/10^{-pH}) - 10^{-pH}]V_0 + C_0V_0/[1 + (10^{-pH}/10^{pK_a})]}{M_{base} + [10^{-pH} - (K_W/10^{-pH})]} \quad (2)$$

where V_{base} = volume of sodium hydroxide added, M_{base} = molarity of sodium hydroxide, C_0 = original molarity of the acid solution, V_0 = original volume of the acid solution to be titrated, and $K_W = 0.9908 \times 10^{-14}$ M^2 (Albert & Sergeant, 1984). The pK_a values thus determined were used to obtain σ^* values by means of eq 3 (Fastrez, 1977).

$$\sigma^* = (4.77 - pK_a)/0.663 \quad (3)$$

The pK_a values of *N*-(benzyloxycarbonyl)glycine, *N*-(methoxycarbonyl)glycine, and *N*-[(phenylmethyl)sulfonyl]glycine were 3.67, 3.63, and 3.40 and thus the σ^* values for the (benzyloxycarbonyl)amino, (methoxycarbonyl)amino, and [(phenylmethyl)sulfonyl]amino substituents were calculated to be 0.60, 0.62, and 0.75, respectively.

Preparation of Enzymes for Thermal Stability Measurements. The phosphonylated β-lactamase was prepared by incubation of the native enzyme (typically 0.5 mL of *ca.* 20 μM) in 20 mM MOPS, pH 7.5, at 25 °C with sufficient inhibitor (**12–17**) to achieve full inactivation in less than 12 h. Methylphosphonyl fluoride was used instead of **12** to generate the methylphosphonyl derivative because of the greater reactivity of the former compound with this enzyme (Rahil & Pratt, 1993). Control studies showed that the loss of activity in the absence of phosphonate was negligible (≤5%) under these conditions. After inactivation, most of the excess inhibitor was removed by two cycles of Amicon Centricon-10 ultrafiltration and redilution in buffer at 4 °C. No enzyme activity was restored by this procedure.

Inhibition of the β-lactamase by 6-β-bromopenicillanic acid was accomplished in 300 μL of a solution containing 20 mM MOPS at pH 7.5, 33.2 μM enzyme, and 48.4 μM bromopenicillin at room temperature. β-Lactamase activity was lost in seconds. The solution was diluted 9 times for the thermal stability experiment. In a similar experiment, 180.6 μM moxalactam produced complete inhibition of the β-lactamase in about 12 h. Again the solution was diluted 9 times in preparation for the thermal stability measurements. In order to test for the possibility of moxalactam being lost from the enzyme upon heating, a solution of the moxalactam-inhibited enzyme was heated to its transition temperature (about 40 °C), in an accelerated run that took 80 min, and then quickly cooled to 25 °C. Aliquots were taken at the beginning, during, and at the end of the run and assayed against benzylpenicillin. There was no sign of any return of activity.

α-Chymotrypsin was inactivated by tris(*p*-nitrophenyl) phosphate, essentially as described by Bender and Wedler (1972). Thus, the phosphate (*ca.* 10 mg) was suspended in 3 mL of Tris buffer (80 mM, pH 7.9, with 0.1 M $CaCl_2$) containing α-chymotrypsin (2.6 mg) and the mixture was magnetically stirred at room temperature. The enzyme activity against *N*-benzoyl-L-tyrosine ethyl ester was assayed spectrophotometrically (256 nm) at suitable intervals by addition of 5-μL portions of the reaction mixture to 0.5-mL aliquots of a solution of the ester (3 mM) in the above Tris buffer containing 25% methanol. After approximately 2 h of reaction, the enzyme activity had fallen to essentially zero. The remaining suspension was centrifuged to remove excess phosphate and the supernatant was dialyzed at 5 °C against 2 × 1 L of 20 mM phosphate buffer at pH 2.0 over a 24-h time period. The resulting enzyme solution was concentrated to about 1 mL by Amicon Centricon-10 ultrafiltration. Prior to dialysis, a sample of the supernatant was monitored spectrophotometrically at 400 nm for *p*-nitrophenolate release; none was observed over a 20-min period.

Thermal Stability Measurements. Typically, each experiment was carried out on a 200-μL sample of a *ca.* 10 μM solution of the enzyme, native or inhibited, in 20 mM MOPS buffer at pH 7.5 in a microcuvette (1-cm light path). A

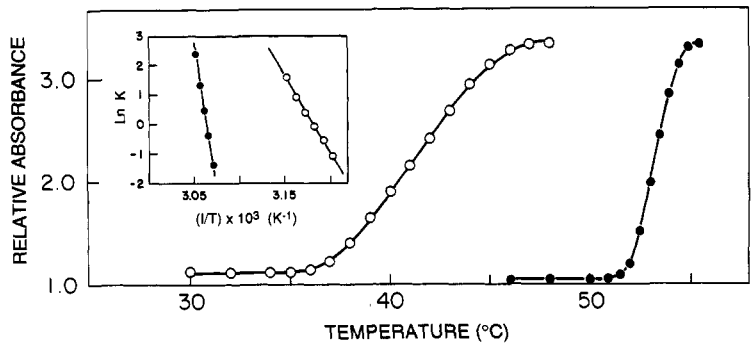


FIGURE 1: Thermal denaturation curves, following the absorption at 280 nm, for the native (○) and phosphonate **15**-inhibited (●) enzymes. Inset are the corresponding van't Hoff plots from which the enthalpies of denaturation were obtained.

Hewlett-Packard Model 8452A diode array spectrophotometer equipped with a temperature controller (Model 89090A) and Peltier junction temperature-controlled cuvette holder with a built-in temperature probe and stirrer was employed to obtain melting curves. The instrument was programmed to increase the temperature (25–90 °C) in 0.5–2 °C increments with 10-min equilibration periods between absorption measurements. After equilibration at each temperature, 200 absorption spectra (from 250 to 350 nm) were collected at a rate of 10 spectra/s. The collected spectra were averaged. Averaged absorbances, usually at 280 nm, were then plotted against temperature and the transition temperature (T_m) was obtained from the inflection points of the denaturation curve, calculated from the first derivatives.

With the assumption of a reversible two-state transition (N ⇔ U), equilibrium constants at any given temperature were calculated from eq 4, where [U] and [N] are the equilibrium concentrations of denatured and native protein, respectively.

$$K = \frac{[U]}{[N]} = \frac{A - A_N}{A_U - A} \quad (4)$$

A is the protein absorbance at the given temperature, A_N is the absorbance of the native protein, and A_U is the absorbance of the denatured protein. In cases where the pre- or posttransitional absorbances varied with temperature, linear extrapolation was used to obtain A_N and A_U at a given temperature within the transition (Hermans, 1965). van't Hoff enthalpies and entropies of the transition were then obtained from linear plots of ln K *vs* $1/T$. Quantitative data on each protein, the native enzyme and each of the inhibited enzymes, was obtained from at least five separate experiments.

RESULTS

Thermal Denaturation of the PC1 β-Lactamase and Derivatives. A thermally induced transition or melting was observed on monitoring the aromatic absorption as the native PC1 β-lactamase and its phosphonyl derivatives were heated in solution, as described in the experimental section (Figure 1). On the assumption of a two-state reversible transition, N ⇔ U, equilibrium constants, K, and thence molar free energies, ΔG, of this transition could be calculated as a function of temperature. Plots of ln K *vs* $1/T$ were linear (e.g., see Figure 1), giving van't Hoff enthalpies, ΔH, and entropies, ΔS, of the transition. The two-state assumption is supported by the identity of values of the transition temperature T_m and ΔH at all wavelengths 250–320 nm, by the linearity of the van't Hoff plots, and by the identity of the T_m values calculated from the inflection points of the melting curves on one hand and from the thermodynamic parameters ($T_m = \Delta H/\Delta S$) on the other. For example, the native enzyme yielded values of T_m of 41.9 ± 1.3 °C by the former method and 41.7 ± 1.1 °C by the latter. Furthermore, variation in the heating rates (from measurements taken at intervals of from 0.5 to 5 °C) led to no systematic variation in T_m or ΔH for either the native or the inhibited enzyme. The melting curves obtained were also unaffected by variation in protein concentration (5–30 μM), indicating that no bimolecular reaction, such as aggregation, influenced the data.

On the other hand, it was also clear that the transition was, in the time frame of the complete experiment, irreversible. The melting curves, such as shown in Figure 1, were not retraced on cooling the sample at the rate at which it had been heated. Further, at temperatures beyond the transition, protein precipitation that was not reversed on cooling was generally seen. Nonetheless, on a shorter time frame the transition appeared to be reversible. For example, in an experiment where faster heating of the native enzyme was used, from 25 to 40 °C, the midpoint of the transition, in 5 °C steps with 10-min equilibration periods, 34% of the enzyme activity was retained on quenching an aliquot of the solution into an assay medium at 25 °C. Thus, a process such as that in Scheme III may well be involved, where an irreversible denaturation presumably involving aggregation occurs beyond a thermodynamically reversible unfolding step (Lumry and Eyring, 1954). Scheme III can lead to complicated melting behavior, depending largely on the (temperature-dependent) relative sizes of k_2 and k_3, but in systems that approximate to either of the two extremes, $k_2 \gg k_3$ and $k_3 \gg k_2$, a useful quantitative treatment is possible. If, at lower temperatures, throughout most of the transition, $k_2 > k_3$, then the two-state thermodynamic model can be applied to experimental data to a good approximation (Manly et al., 1985; Edge et al., 1985; Sturtevant, 1987; Goins & Freire, 1988). The clearest diagnostic of the failure of this approximation is the observation of temperature-scan rate dependent melting curves (Sanchez-Ruiz et al., 1988).

Scheme III

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} U \xrightarrow{k_3} D$$

No detailed study of the thermal denaturation of the PC1 β-lactamase appears to have been published. The unfolding in urea and guanidine hydrochloride has been extensively investigated by Pain and co-workers [for example, see Carrey and Pain (1978), Mitchinson and Pain (1985), and Uversky et al. (1992) and references therein]. At least one thermodynamically stable but catalytically inactive intermediate is observed in these denaturants. There was no evidence for the accumulation of such an intermediate in the thermal denaturation, as revealed by the absorption measurements described above. Calorimetric studies of the unfolding of another class

Table 1: Thermodynamic and Kinetic Data Relating to Various Derivatives of the PC1 β-Lactamase

inhibitor	T_m[a] (°C)	ΔH[a] (kcal/mol)	$\Delta\Delta G_i$ (kcal/mol)	k_i (s^{-1} M^{-1})	k_{cat}/K_M (s^{-1} M^{-1})	substrate
none/wild type	41.6 ± 1.0	111 ± 15	0			
moxalactam	42.6[b]	113[b]	0.22			
6-β-bromopenicillanic acid	45.2[b]	94[b]	–0.71			
12	43.2 ± 0.8	137 ± 21	1.7 ± 0.1	1.7×10^{-5}	2.5	**8**
13	51.8 ± 0.9	241 ± 30	11.3 ± 2.4	0.12	1270	**9**
14	52.9 ± 0.9	218 ± 29	9.8 ± 2.2	0.43	4390	**10**
15	54.0 ± 0.6	339 ± 11	20.8 ± 1.1	2.5	2.0×10^4	**11**
16	54.9 ± 0.1	289 ± 19	16.8 ± 2.0	4.0		
17	54.2 ± 0.4	338 ± 46	19.0 ± 1.7	0.34	2.0×10^4	**11**

[a] Averages of at least five separate determinations except where otherwise noted. [b] Single determinations.

A β-lactamase, *Bacillus cereus* β-lactamase I (Arnold & Viswanatha, 1983; Arriaga et al., 1992), showed complex melting behavior with time-dependent and irreversible phases under the conditions employed.

On the basis of these general considerations and the observations described above, the melting curves obtained in this study were interpreted simply in terms of a reversible two-state thermodynamic model. Melting temperatures (T_m) and enthalpies of the denaturation for the native enzyme, the various phosphonyl derivatives, and two acyl derivatives are given in Table 1.

The molar Gibbs free energy ΔG of denaturation of the native protein at any temperature T is given by eq 5, using the "second standard model of thermodynamics" (Schellman, 1987; Becktel & Schellman, 1987) where ΔC_p for the transition is assumed to be constant over the temperature range under consideration. This is normally a good approximation over the temperature range employed in this work. In eq 5, ΔH_0

$$\Delta G = \Delta H_0 - T\Delta S_0 + \Delta C_p[T - T_m - T \ln (T/T_m)] \quad (5)$$

and ΔS_0 refer to the molar enthalpy and entropy changes, respectively, at a reference temperature, taken here for convenience to be the melting temperature T_m. For a modified protein, eq 6 will be assumed to hold. In this equation, ΔH_i

$$\Delta G_i = \Delta H_i - T\Delta S_i + \Delta C_p[T - T^i_m - T \ln (T/T^i_m)] \quad (6)$$

and ΔS_i refer to the molar enthalpy and entropy changes, respectively, at the melting tempeature T^i_m of the modified protein. At any temperature, therefore, the change in the free energy of denaturation $\Delta\Delta G_i$ produced by the perturbation to the protein structure on formation of the derivative is given by eq 7. To obtain eq 7, the substitutions $\Delta S_0 = \Delta H_0/T_m$, ΔS_i

$$\Delta\Delta G_i = \Delta H_i[1 - (T/T^i_m)] - H_0[1 - (T/T_m)] + \Delta C_p[-\Delta T^i_m - T \ln (T/T^i_m)] \quad (7)$$

$= \Delta H_i/T^i_m$, and $\Delta T^i_m = T^i_m - T_m$ were employed. It is assumed that the subtraction of eq 6 from eq 5 to yield eq 7 essentially cancels out the contributions to ΔG and ΔG_i from the ionization of the buffer and the protein functional groups (Privalov, 1979), except for those protein ionizations affected by the protein modification and therefore involved in the interaction of the phosphonyl moiety with the protein.

Values of $\Delta\Delta G_i$ at 25 °C are given in Table 1. These values were obtained from employment of eq 7 and the assumption of a value of 15 cal deg^{-1} $residue^{-1}$ for ΔC_p for the PC1 β-lactamase and its phosphonyl-derivative, which represents the midpoint of the range of ΔC_p for proteins (12–18 cal deg^{-1} $residue^{-1}$) quoted by Schellman (1987). The PC1 β-lactamase contains 257 residues (Ambler, 1975) and hence ΔC_p = 3.86 kcal/deg. Assumption of either extreme of the range of ΔC_p values does not affect subsequent analysis since the major uncertainty in $\Delta\Delta G_i$ arises from uncertainties in ΔH_0 and ΔH_i.

Thermal Denaturation of α-Chymotrypsin and Derivatives. Inactivation of α-chymotrypsin by tris(*p*-nitrophenyl) phosphate has been shown by Bender and Wedler (1972) to occur in two phases (Scheme IV): first, formation of a phosphate triester **18** with the active-site serine hydroxyl group, followed by an "aging" process where, in an enzyme-catalyzed hydrolysis reaction, a second *p*-nitrophenol is lost, leaving the phosphate diester **19**. A similar "aging" reaction is also well-documented for inactivation of serine proteinases by diisopropyl fluorophosphate (Berends et al., 1959). In general, the initial product **18** is readily reactivated by nucleophiles but the "aged" diester is not. The α-chymotrypsin inactivated by tris(*p*-nitrophenyl) phosphate as described above should, according to Bender and Wedler (1972), be in the "aged" form **19**, because of the ease of hydrolysis of **18**. In agreement with this conclusion, no release of *p*-nitrophenol from the derivative subsequent to the inactivation was observed.

Scheme IV

$$\text{E-OH} + (\text{ArO})_3\text{PO} \rightarrow \text{ArOH} + \underset{\mathbf{18}}{\text{E} - \text{O} - \text{PO(OAr)}_2} \xrightarrow{H_2O} \underset{\mathbf{19}}{\text{E} - \text{O} - \text{PO}_2^-(\text{OAr})} + \text{ArOH} + \text{H}^+$$

Chymotrypsin and its derivatives are known to undergo a readily accessible, reversible, thermal denaturation at low pH (Brandts & Lumry, 1963; Havsteen et al., 1963; Biltonen & Lumry, 1969). We have employed similar conditions in order to compare our results with **19** with those from the literature. Our measurements of melting temperatures and thermodynamic parameters, with relevant data from the literature for chymotrypsin and derivatives, are given in Table 2.

Kinetic Data. Values of k_{cat}/K_M for turnover of **8–11** by the PC1 β-lactamase and of k_i for inhibition of the enzyme by **12–17** are given in Table 1. The new compounds, **13** and **14**, appear to inhibit the enzyme at rates that seem reasonable in view of precedent (Rahil & Pratt, 1991b; 1993); both have amido side chains but are slightly less effective as inhibitors than **15** and **16**.

DISCUSSION

The striking experimental result revealed by these studies was the apparently large stabilization provided to the enzyme by the most effective of the phosphonate inhibitors, **15** and **16**, on forming covalent adducts of structure **2** (Chen et al., 1993). Reaction with these inhibitors increased the melting or denaturation temperature of the enzyme by some 13 °C (Table 1). The question then became one of just how significant, in terms of energy, was this stabilization and

Table 2: Thermodynamic Parameters for Denaturation of α-Chymotrypsin and Derivatives at pH 2.0

protein[a]	method	T_m (°C)	ΔH (kcal/mol)
CT	A_{292}[b]	32.5	90
	$[\alpha]_{365}$[b]	34.7	117
	A_{290}[c]	35.4	102
DIPP-CT[b]	A_{292}	35.3	90
	$[\alpha]_{365}$	35.6	86
AC-CT[b]	A_{292}	31.1	86
	$[\alpha]_{365}$	31.6	87
MNPP-CT[c]	A_{290}	47.4	145

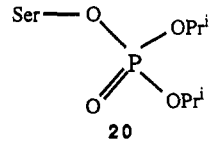
[a] The proteins are α-chymotrypsin (CT), diisopropylphosphoryl-CT (DIPP-CT), acetyl-CT (AC-CT), and mono-*p*-nitrophenylphosphoryl-CT (MNPP-CT). [b] Havsteen et al. (1963). The experiments were carried out in 7 mM HCl and monitored by changes in absorption (A_{292}) or optical rotation ($[\alpha]_{365}$). [c] Present work, carried out in 20 mM phosphate buffer, pH 2.0, and monitored by changes in absorption (A_{290}).

whether it could be related to the question of whether **2** is a good analog of a β-lactamase transition state. In passing, it might be noted that the phosphonyl fluoride **17** yielded a derivative of the same T_m as did **15**. The size of the leaving group in the phosphonylation transition state did not therefore influence the structure of the derivative **2** obtained, i.e., the position of the phosphonate at the active site.

It is well-known of course that covalent modification of a protein, either by chemical means or by mutation, will change its stability, in either a positive or negative sense. Recently, for example, this point has been amply demonstrated with mutant proteins (Goldenberg, 1992). Nevertheless, by definition, in effect, one would expect that a covalently bound transition-state analog would considerably increase the stability of the protein, much more so, for example, than a comparably bound substrate or substrate analog. Enzymes must stabilize transition states, but not necessarily intermediates—and, conversely, transition states and their analogs must stabilize enzymes. Although the experiment cannot be done with a good substrate, because of the lability of its covalent complexes, it is clear that acyl derivatives of the active-site serine of the PC1 β-lactamase do not, in general, produce significant stabilization. This is demonstrated by the melting points of the complexes formed between this enzyme and 6-β-bromopenicillanic acid and moxalactam (Table 1), both of which inhibit by acylation of the active-site hydroxyl group (Cohen & Pratt, 1980; Mazzella & Pratt, 1988).

The striking effects of noncovalently bound transition-state analogs on protein stability have been previously noticed [e.g., Shrake et al. (1989)] although in these cases quantitative analysis, in effect, simply provides an alternative method for determination of the dissociation constant of the ligand—unless the enthalpy of dissociation of a ligand from a protein is comparable to the (large) enthalpy of protein denaturation, the ligand will dissociate prior to protein denaturation (Schellman, 1975, 1976; Pace & McGrath, 1980; Edge et al., 1985).

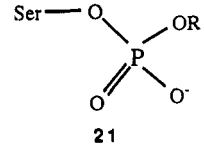
The only previous experiments of this type with covalent inhibitors that we are aware of, but which were not remarked upon at the time for reasons that become clearer below, involve serine hydrolases. These enzymes have long been known to be strongly inhibited by neutral phosph(or/on)yl reagents with the formation of a phosph(or/on)ylated active-site serine hydroxyl group. The classic reagent diisopropyl fluorophosphate of course comes to mind. Although these reagents are often referred to as transition-state analog inhibitors, as noted in the introduction, they seem to have no general stabilizing effect on the enzymes. Thermal data for diisopropylphosphoryl-α-chymotrypsin, for example, are given in Table 2. Little change in T_m is observed with respect to the native protein. On the other hand, however, the diisopropylphosphoryl derivative is reported to be somewhat more stable than the native enzyme toward urea or guanidine hydrochloride (Martin & Bhatnayar, 1966, 1967). These less than dramatic effects probably reflect the point that **20** is *not* a good transition-state analog for chymotrypsin. Not only is it



20

uncharged, but its other specific interactions with the active site are limited. A recent crystal structure of the related diethylphosphoryl-α-chymotrypsin demonstrates this point clearly (Harel et al., 1991). Although the phosphoryl oxygen appears to occupy the oxyanion hole, no interactions with the active-site histidine occur. These drawbacks also apply to phosphonates made more specific to a proteinase with a peptide side chain (Bone et al., 1991). As has been pointed out previously (Rahil & Pratt, 1992), the effectiveness of these neutral phosph(or/on)yl inhibitors of serine hydrolases reflects more the stabilization of the, presumably pentacoordinated, transition state for phosph(or/on)ylation by the enzyme than the nature of the product. Also noticeable from Table 2 is the point that acetylchymotrypsin, the covalent intermediate on the pathway of chymotrypsin-catalyzed acetate ester hydrolysis, is not more stable than the native protein.

More relevant as transition-state analogs are the "aged" products of inhibition of serine hydrolases, of structure **21**, akin to **2**, where one of the alkoxy groups of **20** has been lost by hydrolysis. As mentioned in the introduction, these complexes are thought to be good analogs of the transition states stabilized by these enzymes. Although the effect of



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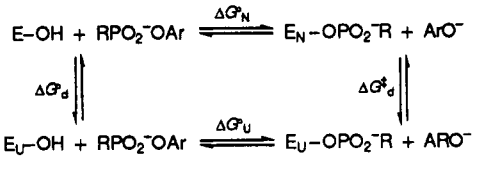
conversion of **20** to **21** has been discussed in terms of its effect on the dissociation constant of the active site histidine (Liang & Abeles, 1987), we are unaware of direct measurements of its effect on the overall stability of the protein. For this reason we prepared the "aged" product formed on inhibition of α-chymotrypsin by tris(*p*-nitrophenyl) phosphate, structure **21** (R = *p*-nitrophenyl), and determined its melting point under the conditions previously reported for the native enzyme and its diisopropylphosphoryl derivative. The greatly enhanced stability of the "aged" complex, T_m = 47.4 °C, ΔT_m = 12 °C, in comparison with the minimal effect of the neutral derivative (Table 2), shows the importance of the negative charge in producing a strong interaction with the enzyme. This, in retrospect, is not surprising since the tetrahedral intermediates of catalysis are believed to be anionic and thus good transition-state analogs must be.

These considerations and results certainly qualitatively and semiquantitatively support the proposition that **2** is a satisfactory transition-state analog of β-lactamase catalysis, since phosphonylation of the PC1 β-lactamase by **15** and **16**, as described above, also has a large stabilizing effect on the enzyme, as reflected in the melting points. Nonetheless, quantitative analysis is necessary to prove the point—all transition-state analogs should, by definition, strongly stabilize their respective enzymes, but the converse is presumably not true.

One method of proceeding further, as described in the introduction, is to compare the free energy change brought about on the binding by the enzyme of the phosphonate inhibitor on one hand and the transition state of the analogous substrate on the other. These energy changes should be the same if the transition-state analog is a good one.

The effect of the phosphonate inhibitors on the stability of the enzyme was obtained by way of their effect on the thermodynamics of the thermal protein melting transition, as shown quantitatively in the Results section, leading to a quantitative estimate of the difference in free energy of thermal denaturation, $\Delta\Delta G_i$, between the native and phosphonate-inhibited enzymes. Although there has been much discussion on the subject of the structure of heat-denatured proteins, and it seems likely that many retain significant structure at temperatures beyond these transitions, little of the detailed tertiary structure of the original protein remains (Tanford, 1968; Privalov, 1979; Privalov et al., 1989; Dill & Shortle, 1991). For the present purposes, it is necessary to assume only that all specific noncovalent interactions between the phosphonate and the enzyme are lost in the denatured state and thus that the structures and energies of the denatured enzymes are the same; this seems likely to be largely true—denatured enzymes in general have little affinity for their substrates and they are poor catalysts. Experimental support for this proposition comes from the observation of essentially identical spectral changes on denaturation of the native and phosphonylated enzymes (Figure 1).

The quantitative rationale for this approach stems from application of the thermodynamic cycle of Scheme V,



where E_N and E_U represent the native and denatured enzymes, respectively, and ΔG°_d and ΔG^*_d represent the standard Gibbs free energies of denaturation of the native enzyme and the transition-state analog complex, respectively; ΔG°_N and ΔG°_U represent the standard free energies of phosphonylation of the active site serine in the native and denatured enzymes respectively. Equation 8 follows from the cycle. If it is assumed

$$\Delta\Delta G_i = \Delta G^*_d - \Delta G^\circ_d = \Delta G^\circ_U - \Delta G^\circ_N \qquad (8)$$

that the free energies of covalent bond formation are the same in the formation of E_N-OPO_2^-R as in E_U-OPO_2^-R, then eq 9 follows, where ΔG^* represents the free energy of non-covalent

$$\Delta G^\circ_U - \Delta G^\circ_N = -\Delta G^* \qquad (9)$$

interaction between the native enzyme and the transition state analog (that between the denatured enzyme and the analog is assumed, above, to be zero). Equations 8 and 9 lead directly to eq 10, which indicates, given the above assumptions, that

$$\Delta G^* = -\Delta\Delta G_i \qquad (10)$$

the free energy of noncovalent interaction between the transition-state analog and the enzyme is equal to the experimentally determinable difference in denaturation free energies.

In order to obtain an estimate of the noncovalent stabilization of the transition state for the analogous substrate, it is necessary to compare the transition states of the uncatalyzed and enzyme-catalyzed reactions (Scheme VI).

Scheme VI

$$E - OH + RCOOAr \xrightarrow{\Delta G^*_E} E - \ddagger$$

$$H_2O + RCOOAr \xrightarrow{\Delta G^*_0} \ddagger$$

Again assuming that the free energy of the covalent chemistry is closely similar between these reactions, eq 11 follows, where

$$\Delta G^* = \Delta G^*_E - \Delta G^*_0 = -RT \ln[(k_{cat}/K_M)/k_{H_2O}] \qquad (11)$$

k_{H_2O} is the second-order rate constant for spontaneous attack of water on the substrate in free solution and ΔG^* is the free energy of noncovalent stabilization of the transition state by the enzyme.

Finally, if **2** is a good transition-state analog, then ΔG^* in eqs 10 and 11 should be the same and hence eq 12 follows.

$$\Delta\Delta G_i = RT \ln [(k_{cat}/K_M)/k_{H_2O}] \qquad (12)$$

An assumption hidden in the above treatment involves the *p*-nitrophenyl leaving group, presumably present in the E – ‡ transition state of Scheme VI but not in **2**. If one assumes the simple turnover sequence of Scheme VII,

Scheme VII

$$E + S \underset{K_s}{\rightleftharpoons} E{\cdot}S \xrightarrow{k_2} E - S \xrightarrow{k_3} E + P$$

where E·S is the noncovalent Michaelis complex and E–S is the acyl-enzyme, $k_{cat}/K_M = k_2/K_s$. It might seem more appropriate, if **2** best resembles the transition state of the deacylation step, to use k_3 [$= k_{cat}$ for this enzyme (Govardhan & Pratt, 1987)], as the relevant kinetic parameter. This procedure, however, would suffer from the uncorrected differences in free energy of interaction between the substrates and enzyme at the acyl-enzyme stage. On the other hand, the employment of k_{cat}/K_M, corresponding to the acylation transition state, assumes that the free energy of interaction of the leaving group, not present in **2**, with the enzyme in the transition state E – ‡ is negligible (or equal to that of $O^{1/2-}$ in **2**). This may in fact be a reasonable assumption since the leaving group is nonspecific to the enzyme, k_{cat}/K_M values for these ester substrates seem not strongly influenced by the nature of the leaving group (Yang Xu & R. F. Pratt, unpublished results), the product phenols do not strongly inhibit the enzyme, and from the crystal structure of the phosphonate complex (see below), the leaving group is probably in a position where little interaction with the enzyme occurs (Chen et al., 1993). Thus eq 12 may not be any poorer an approximation because of this assumption.

The value of k_{H_2O} for substrates **8**–**11** varies because of the different acyl substituents. This can be allowed for by the classical Taft treatment, as expressed in eq 13, where k_{H_2O} is the second-order rate constant for spontaneous hydrolysis of any of the substrates, $k^\circ_{H_2O}$ is the value for the reference compound **8**, σ^* is the Taft parameter for the acyl substituent, determined as described in the experimental section, and ρ^* is a constant describing the sensitivity of the rate constant to the electronic nature of the acyl substituent. A value of 1.6

$$\log k_{H_2O} = \log k^\circ_{H_2O} + \rho^*\sigma^* \qquad (13)$$

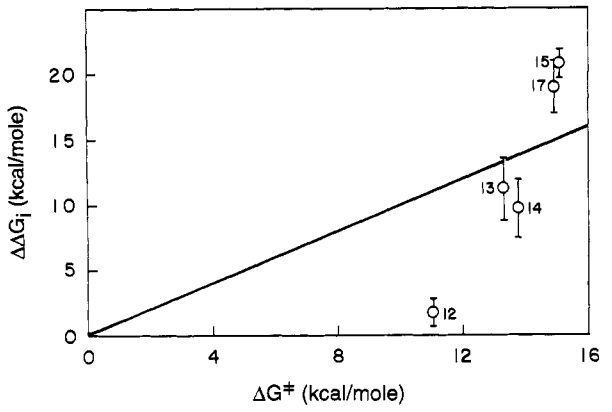


FIGURE 2: Plot showing the relative values of the free energies of noncovalent interaction between the enzyme and the phosphonate on one hand ($\Delta\Delta G_i$) and the transition state on the other (ΔG^*). The latter quantity corresponds to the right-hand side of eq 14. The solid line is drawn of slope 1.0 and through the origin. The numbers associated with the points refer to the phosphonates **12–15** and **17**.

has been taken from the results of Knowlton and Byers (1988) as appropriate for a neutral nucleophile. Changes in this parameter (±1) do not affect the subsequent analysis and conclusions. Another implicit assumption in this treatment that might be noted here for completeness is that the transition-state structure involved in acylation of the enzyme does not change significantly with the intrinsic reactivity of the substrate, or if it does, it is compensated for by a similar change in the transition state for the nonenzymic hydrolysis. Given the rather small change in reactivity involved, this is likely to be a good assumption.

Thus, in final form, eq 12 becomes

$$\Delta\Delta G_i = RT \ln (k_{cat}/K_M) - 2.303\, RT(\log k^{\circ}_{H_2O} + \rho^*\sigma^*) \quad (14)$$

A plot of $\Delta\Delta G_i$ *vs* the right-hand side of eq 14 should yield a straight line of unit slope, passing through the origin if the approximations made in achieving eq 14 are reasonable and if **2** is in fact a good approximation to a transition-state structure. This plot is shown as Figure 2, giving the positions of the experimental points with respect to the theoretical line.

With perspective, the fit can be seen as good, with the points for compounds **9–11** (**13–15**), which, in principle, could be anywhere on the field, in fact clustered close to the line. Since their deviations from the line are both positive (**15**) and negative (**13** and **14**), it seems likely that no single factor or assumption is responsible for them. In these cases, therefore, the free energy of noncovalent interaction of the phosphonates with the active site of the enzyme in **2** is closely similar to the free energy of stabilization of the related transition states **3**. This can be taken as quite persuasive evidence that **13–15** are transition-state analog inhibitors in the full sense of the term.

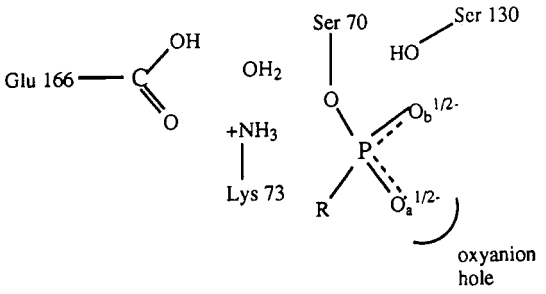
In contrast to these results, the point for **8** (**12**) lies well below the theoretical line. Such a large deviation could arise from a much smaller interaction of the phosphonate with the active site, i.e., a much lower T_m for **2**, than of the transition state of **8** hydrolysis, or a much more effective hydrolysis of **8** than would be expected from the active-site interactions of **12**. The most likely explanation is that nonspecific hydrolysis of **8** by the enzyme has not been completely controlled for (it should be noted here that, under the conditions employed, the enzyme-catalyzed reaction was only 13% ± 5% above background). Actually, the predicted k_{cat}/K_M value for **8**, on the basis of the theoretical line and the measured T_m of the enzyme inhibited by **12**, would be *ca.* 3×10^{-7} s^{-1} M^{-1}, which would be impossible to measure at any feasible enzyme concentration against the background hydrolysis rate at pH 7.5. It seems likely, therefore, that the k_{cat}/K_M value for **8** is artifactual. A line through all the points of slope *ca.* 4 and large negative intercept would not appear to have likely physical meaning.

We believe, therefore, that these results indicate that this general method of quantitative assessment of covalently bound transition-state analogs is valid and useful. The least easily controlled aspect lies in the selection of the range of analogs to be employed. Too small a range in $\Delta\Delta G_i$ as seen here in **9–11** (**13–15**) leads to uncertainties in the linear correlation, while too large a range, as seen here with **8** (**12**), can lead to practical problems. It is useful in these studies to choose structural perturbations far enough away from the reaction center so as to have little influence on the nonenzymic reaction rates (Bartlett & Marlowe, 1983), although, as shown in this work, correction for changes in the latter can be done. Obviously, side chains with substitutions further from the reaction center could have been chosen here, but much trial-and-error experimentation would have been needed to assemble a group with a sufficient span of $\Delta\Delta G_i$. Any other method of determination of protein stability could presumably also be employed, for example, urea or guanidine hydrochloride denaturation. Since the requirement is only that all significant, i.e., transition-state specific, interactions between the transition-state analog and the protein be lost in the denatured state, partial unfolding, e.g., of one domain, might in some cases be sufficient.

Of some interest is the impressively large increase in the enthalpy of protein denaturation brought about by these transition-state structures, of the order of 200 kcal/mol in the enzymes inhibited by **15** and **16** (Table I) and apparent visually in the steepness of the thermal denaturation curves (Figure 1). As is often observed in aqueous solution, the effect on the Gibbs free energy is much less because of extensive entropy compensation. Nonetheless, the changes in ΔH and ΔS are striking. A visual comparison of the crystal structures of the native and inhibited enzymes (Chen et al., 1993) gives no obvious indication of their source. Indeed, the structures of the two proteins are remarkably similar. The explanation may lie with differences between the dynamics of the proteins in solution and their interactions with water.

A β-Lactamase Transition State. Given, as concluded above, that the covalent complex between the phosphonate **15** and the PC1 β-lactamase does closely resemble a β-lactamase transition state involved in the turnover of an acyclic substrate such as **11**, there arises the question as to just which of the transition states of the reaction it resembles—β-lactamase catalysis involves two acyl transfer steps, corresponding to the formation and subsequent hydrolysis of an acyl-enzyme intermediate, where each step presumably involves the formation and breakdown of a tetrahedral intermediate; hence four transition states are passed through during the complete reaction [in *p*-nitrophenyl esters, at least, the acylation reaction may be concerted, with a single tetrahedral transition state (Ba-Saif et al., 1987; Hengge, 1992), but in view of the position of functional groups in the crystal structure, discussed below, this distinction is not important here]. The major functional groups of the active site appear in the crystal structure of the phosphonate complex (Chen et al., 1993) as represented schematically in **22**. The important feature seems to be that the functional groups of the active site (including the occluded water molecule) are all clustered closer to the oxygen of Ser-70 than to the O_b of the phosphonate. This, as pointed out

in the introduction, is contrary to what is seen in analogous serine proteinase structures.



22

The clustering of the catalytic groups in **22** suggests an "outer" transition state, either (a) formation of the tetrahedral intermediate in acylation or (b) breakdown of the tetrahedral intermediate in deacylation, rather than an "inner" transition state, either (c) breakdown of the tetrahedral intermediate in acylation or (d) formation of the tetrahedral intermediate in deacylation, where they should be clustered around the phosphonate O_b. It should be noted here that there is currently considerable belief in an unsymmetric mechanism for β-lactamases (Herzberg & Moult, 1991; Escobar et al., 1991), where the mechanisms (and hence distributions of functional groups in the transition state) of the acylation and deacylation steps are different, again in contrast to serine proteinases, where a symmetric mechanism is generally accepted.

In terms of the structure **22**, alternative (b) would involve general acid catalysis of O-Ser-70 departure by one of the following: Lys-73-NH_3^+ (assuming it to be protonated, as shown in **22**), the water molecule, aided by Glu-166 (assumed to be protonated, as shown; Lys-73 in this mechanism would provide orientational and electrostatic catalysis) and Ser-130-OH. This interpretation would be, however, contrary to most current thought since it would imply that the incoming water molecule required for deacylation comes from "outside" the active site rather than from "inside" (the occluded water molecule).

Alternative (a), despite the absence of a leaving group on phosphorus, would involve a complementary scenario—general base catalysis of attack by HO-Ser-70 by either Lys-73 in neutral form (Strynadka et al., 1992), the water molecule aided by anionic Glu-166 (Lamotte-Brasseur et al., 1991), or HO-Ser-130. The first of these can be criticized on the basis of the difficulty of correlating the properties of a neutral lysine amine group with the group responsible for the ascending limb of a classical class A pH–rate profile (Knap & Pratt, 1991; Fink, 1992) and the significant activity of a Lys-73 Arg mutant of one class A enzyme—certainly much greater than a Glu-166 mutant (Gibson et al., 1990). The second would presumably be criticized by those who believe that Glu-166 is not involved in the acylation step on the basis of the fast acylation but slow deacylation of Glu-166 mutants (Escobar et al., 1991); no direct measurements of acylation rates on these mutants have yet been published, however, and it is rather difficult to see how mutation of Glu-166, positioned as it is, could not have some effect on acylation rates.

Given the above choices, the combination of transition state (a) and proton transfer catalyzed by Glu-166 and the water molecule seems most likely to the present authors. Certainly the structure **22** does not support any mechanism involving direct contact between Glu-166 and the substrate (Gibson et al., 1990; Knap & Pratt, 1991). The requirement for such a mechanism would have been motion of Glu-166 in toward the bound inhibitor and this has not occurred. Ser-130 also seems best placed to provide a proton to a leaving group in the position of O_b, as others have suggested (Lamotte-Brasseur et al., 1991; Strynadka et al., 1992). The crucial unknown at present is the state of dissociation of Glu-166 and Lys-73 in the active free enzyme and in **2**. With respect to the latter, it is possible that the structure of **2**, determined at pH 8.0 (Chen et al., 1993), contains one proton less than the real transition state [the pH–rate dependence of the PC1 β-lactamase is associated with the dissociation of functional groups of pK_as around 7.5 and below 4.0 (Anderson & Pratt, 1983)]. We do find that the thermal transition temperature of **2** is greater at lower pH, but so too is k_{cat}/K_M; eq 14 may well therefore still hold.

REFERENCES

Albert, A., & Sergeant, E. P. (1984) *The Determination of Ionization Constants of Acids and Bases*, 3rd ed., Chapter 2, Chapman & Hall, New York.

Aldridge, W. N., & Reiner, E. (1972) *Enzyme Inhibitors as Substrates*, American Elsevier, New York.

Ambler, R. P. (1975) *Biochem. J. 151*, 197–218.

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

Arnold, L. D., & Viswanatha, T. (1973) *Biochim. Biophys. Acta 749*, 192–197.

Arriaga, P., Menendez, M., Villacorta, J. M., & Laynez, J. (1992) *Biochemistry 31*, 6603–6607.

Ashani, Y., & Green, B. S. (1982) in *Chemical Approaches to Understanding Enzyme Catalysis: Biomimetic Chemistry and Transition State Analogs* (Green, B. S., Ashani, Y., & Chipman, D., Eds.) pp 169–188, Elsevier, Amsterdam.

Bartlett, P. A., & Marlow, C. K. (1983) *Biochemistry 22*, 4618–4624.

Ba-Saif, S., Luthra, A. K., & Williams, A. (1987) *J. Am. Chem. Soc. 109*, 6362–6368.

Becktel, W. A., & Schellman, J. A. (1987) *Biopolymers 26*, 1853–1857.

Bender, M. L., & Wedler, F. C. (1972) *J. Am. Chem. Soc. 94*, 2101–2109.

Berends, F., Posthumus, C. H., van der Sluys, I., & Deierkauf, F. A. (1959) *Biochim. Biophys. Acta 34*, 576–578.

Bernhard, S. A., & Orgel, L. E. (1959) *Science 130*, 625–626.

Biltonen, R., & Lumry, R. (1969) *J. Am. Chem. Soc. 91*, 4251–4256.

Bodanszky, M., & Bodanszky, A. (1984) *The Practice of Peptide Synthesis*, Springer-Verlag, Berlin.

Bone, R., Sampson, N., Bartlett, P. A., & Agard, D. A. (1991) *Biochemistry 30*, 2263–2272.

Brandts, J., & Lumry, R. (1963) *J. Phys. Chem. 67*, 1484–1494.

Carrey, E. A., & Pain, R. H. (1978) *Biochim. Biophys. Acta 533*, 12–22.

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

Cohen, S. A., & Pratt, R. F. (1980) *Biochemistry 19*, 3996–4003.

Dill, K. A., & Shortle, D. (1991) *Annu. Rev. Biochem. 60*, 795–825.

Edge, V., Allewell, N. M., & Sturtevant, J. M. (1985) *Biochemistry 24*, 5899–5906.

Escobar, W. A., Tan, A. K., & Fink, A. L. (1991) *Biochemistry 30*, 10783–10787.

Fastrez, J. (1977) *J. Am. Chem. Soc. 99*, 7004–7013.

Fink, A. F. (1992) *Chemtracts—Biochem. Mol. Biol. 3*, 395–399.

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

Goins, B., & Freire, E. (1988) *Biochemistry 27*, 2046–2052.

Goldenberg, D. P. (1992) in *Protein Folding* (Creighton, T. E., Ed.) pp 353–403, W. H. Freeman, New York.

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

Hanson, J. E., Kaplan, A. P., & Bartlett, P. A. (1989) *Biochemistry 28*, 6294–6305.
Harel, M., Su, C.-T., Frolow, F., Ashani, Y., Silman, I., & Sussman, J. L. (1991) *J. Mol. Biol. 221*, 909–918.
Havsteen, B., Labouesse, B., & Hess, G. P. (1963) *J. Am. Chem. Soc. 85*, 796–802.
Hengge, A. (1992) *J. Am. Chem. Soc. 114*, 6575–6576.
Hermans, J., Jr. (1965) *Methods Biochem. Anal. 13*, 81–111.
Herzberg, O., & Moult, J. (1991) *Curr. Opin. Struct. Biol. 1*, 946–953.
Jencks, W. P., & Carriuolo, J. (1960) *J. Am. Chem. Soc. 82*, 1778–1785.
Knap, A., & Pratt, R. F. (1991) *Biochem. J. 273*, 85–91.
Knowlton, R. C., & Byers, L. D. (1988) *J. Org. Chem. 53*, 3862–3865.
Kossiakoff, A. A., & Spencer, S. A. (1981) *Biochemistry 20*, 654–664.
Kraut, J. (1977) *Annu. Rev. Biochem. 46*, 331–358.
Lamotte-Brasseur, J., Dive, G., Dideberg, O., Charlier, P., Frère, J.-M., & Ghuysen, J.-M. (1991) *Biochem. J. 279*, 213–221.
Liang, T.-C., & Abeles, R. H. (1987) *Biochemistry 26*, 7603–7608.
Lienhard, G. (1973) *Science 180*, 149–154.
Lumry, R., & Eyring, H. (1954) *J. Phys. Chem. 58*, 110–120.
Manly, S. P., Matthews, K. S., & Sturtevant, J. M. (1985) *Biochemistry 24*, 3842–3846.
Martin, C. J., & Bhatnagar, G. M. (1966) *Biochemistry 5*, 1230–1241.
Martin, C. J., & Bhatnagar, G. M. (1967) *Biochemistry 6*, 1638–1650.
Mazzella, L. J., & Pratt, R. F. (1989) *Biochem. J. 259*, 255–260.
Mitchinson, C., & Pain, R. H. (1985) *J. Mol. Biol. 184*, 331–342.
Pace, C. N., & McGrath, T. (1980) *J. Biol. Chem. 255*, 3862–3865.
Phillips, M. A., Kaplan, A. P., Rutter, W. J., & Bartlett, P. A. (1992) *Biochemistry 31*, 959–963.
Pratt, R. F. (1989) *Science 246*, 917–919.
Pratt, R. F., & Loosemore, M. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4145–4149.
Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167–241.
Privalov, P. L., Tiktopulo, E. I., Venyaminov, S. Y., Griko, Y. V., Makhatadze, G. I., & Khechinashvili, N. N. (1989) *J. Mol. Biol. 205*, 737–750.
Rahil, J., & Pratt, R. F. (1991a) *J. Chem. Soc., Perkin Trans. 2*, 947–950.
Rahil, J., & Pratt, R. F. (1991b) *Biochem. J. 275*, 793–795.
Rahil, J., & Pratt, R. F. (1992) *Biochemistry 31*, 5869–5878.
Rahil, J., & Pratt, R. F. (1993) *Biochem. J. 296*, 389–393.
Sanchez-Ruiz, J. M., Lopez-Lacomba, J. L., Cortijo, M., & Mateo, P. L. (1988) *Biochemistry 27*, 1648–1652.
Schellman, J. A. (1975) *Biopolymers 14*, 999–1018.
Schellman, J. A. (1976) *Biopolymers 15*, 999–1000.
Schellman, J. A. (1987) *Annu. Rev. Biophys. Biophys. Chem. 16*, 115–137.
Shrake, A., Fisher, M. T., McFarland, P. J., & Ginsberg, A. (1989) *Biochemistry 28*, 6281–6294.
Stroud, R. M., Kay, L. M., & Dickerson, R. E. (1974) *J. Mol. Biol. 83*, 185–208.
Strynadka, N. C. J., Adachi, H., Jensen, S. E., Johns, K., Sielecki, A., Betzel, C., Sutoh, K., & James, M. N. G. (1992) *Nature 359*, 700–705.
Sturtevant, J. M. (1987) *Annu. Rev. Phys. Chem. 38*, 463–488.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121–275.
Uversky, V. N., Semisotnov, G. V., Pain, R. H., & Ptitsyn, D. B. (1992) *FEBS Lett. 314*, 89–92.
Waley, S. G. (1974) *Biochem. J. 139*, 789–790.
Waley, S. G. (1992) in *The Chemistry of β-Lactams* (Page, M. I., Ed.) pp 198–228, Blackie, Glasgow, Scotland.
Wolfenden, R. (1972) *Acc. Chem. Res. 5*, 10–18.